METABOLIC INTERRELATIONS

Transactions of the Third Conference

New York, N Y January 8 9, 1951



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Sponsored by
JOSIAH MACY, JR FOUNDATION

Price $4 00

Printed in the United States of America
by Progress Associates, Inc, Caldwell N J

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FRANK FREMONT SMITH

Medical Director

It gives me great pleasure to welcome you to the Third Conference on Metabolic Interrelations

The regular members who form the nucleus of this group are already well acquainted with the nature and purpose of the Foundation's Conference Program but for the benefit of the guests present I would like to take a few minutes to outline what it is that the Foundation hopes to accomplish by these meetings

We are interested first of all in furthering knowledge about metabolic interrelations and to this end you have been brought together to exchange ideas experiences data and methods In addition to this particular goal however there is a *further and perhaps more fundamental aim* which is shared by all our conference groups This is the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and a very urgent one the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another channels must be established for the most relevant dissemination of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science is a serious obstacle to scientific progress Particularly is it so in medicine that the limited view through the lens of one discipline is no longer enough For example today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation *At the other extreme medicine is certainly a social* science and through mental health must be concerned with economic and social questions The answer then is not further fragmentation into increasingly isolated specialties disciplines and departments but the integration of science and scientific knowledge for the enrichment of all branches *This integration we feel can be encouraged by providing opportunities* for a multiprofessional approach to given topics

Although the fertility of the multidiscipline approach is recognized adequate provision is not made for it by our universities scientific societies

and journals And perhaps, if we are honest we must admit the presence of other hindering factors Partly semantic in nature, they may also to some degree be psychological Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar By making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers

Thus our meetings are in contrast to the usual scientific gatherings to which you are accustomed They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice For this reason we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting Our hope is that the participants will not come prepared to defend a single point of view but will take advantage of this meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories

We have now thirteen groups functioning under the Conference Program The following topics are covered Adrenal Cortex Aging Biological Antioxidants Blood Clotting Blood Pressure Connective Tissues Consciousness Cybernetics Infancy and Childhood Liver Injury Metabolic Interrelations Nerve Impulse, and Renal Function When a new conference is organized the Chairman in consultation with the Foundation selects fifteen scientists to be the nucleus of the group and every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting, but for the purpose of promoting full participation by all members and guests attendance at any meeting is limited to twenty five It is inevitable that in no topic can we possibly include more than a small fraction of the key investigators in the field, and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include

As you know the transactions of these meetings are recorded and published This is done because the Foundation, in addition to its interest in the problem of communication between scientists believes that conveying to those in other fields who are concerned with science the essential nature of scientific research is also an important problem in communication Logic is a vital aspect of science, but equally essential is the intuitive or creative aspect In my opinion, research is as creative as the painting of a portrait or the composing of a symphony Although logic is, of course, necessary in order to rearrange, to test, and to validate, research

thrives on creativity which has its source in unconscious, nonrational processes Unfortunately, however, in the finished products which we present to the world through our research reports this integral part of scientific endeavor is shriveled by the cold white light of logic By preserving the informality of our conferences in the published transactions, we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays

From what I have said it must be apparent that these conferences and the published transactions are an experiment You are a part of that experiment, and the success of it will be measured by how much you, as a participant gain from the conference We hope that at this meeting you will feel the freedom inherent in the scientific method, and that if means occur to you by which we may improve our conference procedure you will share them with us

RESORPTION OF BONE[1]

WILLIAM BLOOM

From the Department of Anatomy University of Chicago Chicago Ill

Armstrong If there are no questions about procedure I will ask Dr Bloom to introduce his subject of "Resorption of Bone

Bloom Resorption of bone occurs normally in the remodelling which takes place during growth This is usually a relatively slow process which continues over a long period of time and is therefore difficult to follow in detail Extremely widespread and extremely rapid resorption of bone occurs in the accessory bone of the marrow of laying birds in connection with the calcification of the egg shell As the resorption of bone in these animals may take place within 6 to 20 hours depending on the species of bird the process is so rapid that cellular and other changes can be followed much more readily

The fact that in growing rats on a very high calcium and phosphorus intake the secondary spongiosa is much longer than in rats on normal mineral intake indicates a failure of resorption to keep pace with bone formation

Resorption of bone occurs after physical injury to bone as for instance after irradiation where there is severance of the primary spongiosa from the calcifying cartilage It may be found under areas of localized pressure It takes place around metastases to bone and in certain obscure chronic diseases (osteomalacia and Paget s disease) It also occurs in the newly formed bone of the callus in the healing of fractures and it is seen in certain infections of bone and after large amounts of parathyroid hormone either from injection of the extract or from tumors of the gland

Effects of Parathyroid Hormone on Resorption of Bone

For some years we have been studying the effects of parathyroid hormone on the resorption of bone Shortly after the injection of toxic

[1]This work was aided by grants from the Division of Research Grants and Fellowships of the National Institutes of Health Public Health Service and the Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

[2]Bloom W Bloom M A and McLean F C Calcification and Ossification. Medullary Bone Changes in the Reproductive Cycle of Female Pigeons *Anat Rec* 81 443–466 (1941)

amounts into rats there is a breakdown of bone, particularly of the s| iosa of the metaphysis We feel certain that in the very first stages o process osteoclasts are not mobilized and accordingly cannot partic in the large scale destruction of bone Later (9 12 hour stages), the prominent in the areas of bone destruction

At last year s meeting I mentioned the experiments of Barnicot which he grafted parathyroid and other endocrine glands to bits of which he implanted into the cerebrum of mice He found that the resorption of the grafted bone only when it carried parathyroid g He also found that with all types of grafts there is a considerable formation of bone At the time I mentioned this work I reported Dr Chang in my laboratory was repeating it and that she also founc remarkable resorption of bone that Barnicot had discovered[3] Dr C simplified Barnicot s experiment by substituting intact parietal bone his bone grafts and placing her tissue grafts as close to the parietal as possible, beneath the connective tissue covering the bone In additic making autogenous and homogenous grafts of parathyroid gland in mice and rats she made many controls with implants of thyroid, ant lobe of the pituitary adrenal cortex, gastric mucosa, pancreas uri bladder mucosa, testis, blood clot tendon, cartilage, various types of t bone marrow, spleen, lymph node cartilage silk, soft paraffin There a marked resorption of bone beneath the parathyroid grafts in both and mice As seen in section, the resorption begins at the poles of the g and the excavation may be for a considerable depth after 4 to 8 d whereas the bone immediately beneath the graft may show little resorp at this time This may possibly be due to interruption of blood suppl the gland, but at any rate after 10 days or so the maximal excavatio beneath the graft and after 18 to 20 days the parietal bone is comple resorbed beneath the graft

In the relatively slow resorption of bone under the graft, osteoclasts prominent and form an almost continuous layer on the surface of the t which is being eroded

New bone formed at the periphery in moderate amounts, and it was e in all cases to distinguish between the new bone and the border of original parietal bone and to see whether resorption had taken place or

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[3]Barnicot N A The Local Action of the *Parathyroid and Other Tissues on* E in Intracerebral Grafts *J Anat* 82 233 249 (1948)

Traces of resorption of the bone occurred in some of the other implants with the silk thyroid gastric mucosa but the amount of erosion of the bone was small and probably is not permanent In only one case that of the soft paraffin a small amount of cartilage developed in the connective tissue around the foreign substance or tissue

It seems to me that on the basis of Barnicot s observation and Chang s corroboration of its correctness we must come to the conclusion that a potent material for the resorption of bone is given off by the parathyroid gland and that this acts directly on bone Surely it is difficult to reconcile this localized resorption in the vicinity of the graft with any theory which holds that the action of the hormone on bone is secondary to a systemic reaction This does not exclude the possibility or probability that the parathyroid hormone may affect connective tissue in general (as well as bone) Nor does it explain why the action of the hormone should be so effective in the zone of growing bone

Histologic Demonstration of Functional Activity of Cells in Bone

I would like to consider now some of the findings which Dr Heller has made during the past year[5] She applied the Hotchkiss stain to bits of frozen dried bone and developed a useful modification of the v Kossa method for demonstrating bone salt in developing and disintegrating bone matrix The Hotchkiss periodate leucofuchsin method demonstrates glycogen and apparently glycoproteins The method as applied by Dr Heller works only on frozen dried material Aqueous fixatives do not preserve the glycoproteins Alcohol fixation although better than aqueous fixatives changes the composition of bone matrix more than freezing drying does

Dr Heller found that in completely formed bone the ground substance is not stained or at most is an exceedingly pale pink In bone being laid down and in bone being resorbed rapidly there were changes in bone matrix which appeared as staining of varying intensities ranging from a definite red color to a very deep fuchsin red Gersh and Catchpole who applied this method to a variety of processes in the connective tissue interpreted the intense staining as indicating a low degree of polymerization or aggregation of glycoproteins while a high degree of polymerization was reflected in pale staining of the matrix owing to the lack of free aldehyde groups to combine with the fuchsin In our photomicrographs it is clearly shown that whatever the chemical interpretation of the process a marked change in the ground substance of bone around the bone cells takes place

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[5]Heller M Occurrence of Possibly Secretory Granules in Osteogenic Cells *Anat Rec* **106** 204 (1950)

after the administration of large amounts of parathyroid hormone The liberation of osteocytes, made possible by the dissolution of the bone matrix, can be followed easily

A fortunate accident enabled Dr Heller to develop a delicate silver method for *staining bone salt*[6] *This method, based on a dilute silver nitrate* solution (0 25%) and exposure for only a few minutes to ultraviolet light, permitted her to distinguish, after injection of parathyroid extract, between the matrix around the bone cells and that which was a slight distance away *This intense blackening of matrix* in the zone of resorption she interprets as indicating a greater solubility of bone salt and hence a greater reactivity with silver nitrate

There are two points in her material which should be emphasized first the lacunae which at this time in the routine sections are found to be optically negative (so called "empty lacunae") are filled with a material which stains deep red by the Hotchkiss and black with the dilute silver *method Secondly, the change in the matrix as demonstrated by the Hotch*kiss and the dilute silver methods is essentially co extensive

Origin of Osteoblasts and Osteoclasts

On the basis of our experience in the rapid formation and destruction of bone we have come to the conclusion that the cells concerned in these processes are the same cells in different functional states That is, when bone is developing in marrow the *reticular cells* can turn into *osteoblasts* and these into osteocytes When bone is being destroyed, whether by parathyroid hormone in mammals or by the stimulus producing the rapid changes in the accessory bone during the egg laying cycle in birds, the liberated osteocytes and osteoblasts form osteoclasts These, in turn, can go back to osteoblasts or can settle down as reticular cells I should like to stress that this conclusion, which we reached on the basis of a study of rapid cellular transformations in the birds and after parathyroid hormone, was foreshadowed many years ago by Koelliker's observations based on slowly progressive changes in bone over a period of weeks

Although most people speak glibly of osteoblasts forming bone and osteoclasts *destroying it, we have little evidence proving that* these cells perform these functions although it is probable that they do On the whole osteoblasts seem to be universally present when bone is being formed rapidly and it is quite likely that they are the cells responsible for laying

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[6]Heller M , McLean F C and Bloom W Cellular Transformations in Mammalian Bones Induced by Parathyroid Extract *Am J Anat* 87 315 348 (1950)

down the matrix of bone just as the closely related fibroblasts make collagenous fibers and reticular cells make reticular fibers As mentioned above we have seen early stages of bone destruction after parathyroid hormone in which it would seem that the breakdown of bone can take place before osteoclasts are mobilized in great numbers

We have been studying the possible role of collagen splitting enzymes and depolymerizing enzymes on bone destruction The results to date seem to indicate that this is a profitable field of study but I would not care to say any more about the work at this time

Conference Discussion

Armstrong Thank you very much Dr Bloom for your presentation

We have plenty of time to explore this subject very thoroughly There will be a number of questions I am sure in the minds of all of us I shall start the discussion by asking the first question

Were these grafts of the parathyroid gland vascularized that is did they take?

Bloom Yes Both autogenous and homogenous grafts took very well The finding that the homogenous grafts took so nicely saved a great deal of time and work Although they were exceedingly small they could be dissected out beforehand and then put into place in the anesthetized animals

Follis Do you consider that this new bone formation was both from periosteum as well as the bone itself?

Bloom You mean in the case of the bone grafts?

Follis Yes

Bloom I think we have to distinguish between two types of bone formation in these grafts Most of the new bone arises as a result of irritation of the periosteal bone In addition there was some that seemed to develop in the granulation tissue around the graft In the case of spleen and in one of the bone marrow grafts a little bit of bone developed inside the graft—I would guess from the reticular cells of those organs

But specifically to answer your question I think most of the bone comes from the periosteum of the parietal bone However in the case of the urinary bladder for instance I am sure that some of it also came from granulation tissue of the periosteum or in the periosteum Does that help?

Follis Yes

Hodge Dr Bloom I should like to ask this question In the test where you found a directional effect growth more to one side or more toward one pole than another is there any chance that a circulation of blood or lymph is going on in that direction?

Bloom This is not an explanation but is a guess This (drawing at blackboard) is the parietal bone with the graft above it Actually there is a trench along the perimeter of the graft The only thing I could think of was that something was interfering with the blood supply in and beneath the middle of the graft perhaps affecting this portion of the gland in some way At first there might be diffusion only from the periphery of the graft and then after a week or so perhaps with complete vascularization diffusion would be in all directions In this condition the greatest concentration would be beneath the center of the graft and it is here that the excavation of bone is greatest

I have a few slides showing the effect of radiation on the metaphysis Strangely enough it hits the part of the spongiosa where there are the fewest cells

Armstrong I should like to hear some discussion perhaps from Dr Hendricks who I think has made the greatest single advance in our knowledge of the constitution of the mineral salt I wonder if he would give us the result of any of his speculations with regard to the differences in the uptake of the silver nitrate stain which Dr Bloom has observed I take it that in the cycle of bone resorption the uptake of the silver by the bone salt differs

Maybe Dr Bloom can express this point a little more definitely so Dr Hendricks can see what I am driving at The whole question comes down to What is the mechanism of the silver stain of the bone salt?

Bloom I wish it were some reagent other than silver nitrate The best way that I have been able to visualize it is this If a relatively large aggregate of bone salt and very small aggregates are stained for a short time as Dr Heller has done with her dilute silver nitrate method and are then examined with transmitted light it is possible that the small ones would appear black because the silver nitrate has penetrated most of them But the silver nitrate could not penetrate most of the large aggregate but would only stain its surface so that it would accordingly appear yellowish or gray by transmitted light If the stain is demonstrating phosphate and carbonate it may well be that in one case it is demonstrating an exceedingly fine powder as against scattered phosphate or carbonate on the surface of a very large grouping

I might add that we have had great difficulty in staining enamel with

silver nitrate and I think it was because of that experience that we have been thinking along these lines

I just want to repeat once more that I do wish it were something other than silver nitrate

Hendricks I should like to avoid giving an immediate answer for the reason that probably fifteen minutes would be required to cover the question

Armstrong I wish you would discuss this question

Hendricks In view of the continuity of the subject this might not be the appropriate time for the discussion

Armstrong I think it is

Hendricks It would seem to me that Dr Bloom s conclusion is a perfectly reasonable one Enamel in one sense is a very compact material with coarse crystals Surface area measurements by Dr Hodge and others show that the apparent size of the particle is of the order of 0 5 microns whereas in much of the bone salt material of bone itself the particle would be of the order of a fiftieth of that size That would constitute enough difference in external surface to lead to differences in reaction with a silver salt, which reacts both with carbonate and phosphate and ultimately to deposition of metallic silver following irradiation

Armstrong You are satisfied then that the reaction could be on the basis of particle size and that we do not need to bring into consideration differences of composition or variations of absorbed substances on the particles?

Hendricks It does not look as though any additional point would be required but that it would be simply a question of how much carbonate phosphate surface is available to the stain which I understand will both be a question of particle size and a question also of avenues by which the stain can approach the particle

Bloom Yes Silver unfortunately has a predilection for reducing on surfaces It is widely used for many other purposes that I am sure have nothing to do with phosphate and carbonate

Follis Is there any chance of reducing groups being made available as the matrix is being destroyed?

Bloom Possibly

Follis Groups that would reduce the silver locally?

Bloom That is possible

Hendricks Is the silver reduced before the staining or after? The silver is reduced later by the light isn t it?

Bloom By the light

Follis But you can get staining if you do it in the dark?

Bloom And especially if you use a concentrated solution for a long time

It is important to remember that these changes in the matrix both with Hotchkiss stain and with silver nitrate are apparent following parathyroid hormone injection hours before osteoclasts are present in any great numbers I think that this time relationship is very important

Follis I wonder if you would comment on the possible importance of humoral factors in bone destruction

Bloom You mean as a general mechanism?

Follis Yes that is the concentration of calcium and phosphorus

Bloom I do not think you can avoid that If I remember correctly—Dr McLean can check me on this—in a rat on a very high calcium and phosphate diet the metaphysis is very much longer than that in an animal on a normal diet

McLean I am not quite sure what question you are asking There are two answers I think One is the effect of low calcium and phosphorus on producing resorption and the other is the effect of high calcium and phosphorus on preventing resorption

Follis The point I should like to hear Dr Bloom comment on is How much effect do you think the local concentrations of calcium and phosphorus that is the humoral concentrations have to do with stimulating bone destruction?

Bloom This is the point I was going to make if my memory had served me correctly

Follis That is you think at the moment that the parathyroid hormone locally stimulates the destruction of bone by the osteoclasts I was wondering how much you felt in the absence of that just lowering the concentration of calcium and phosphorus locally would have to do with the destruction of bone?

Bloom I do not know At least I cannot see any other explanation for the work of Barnicot Something comes out of the parathyroid which affects the bone near it Secondly, the length of the metaphysis in a growing rat on a normal diet is much shorter than that in a rat on a very high calcium and phosphorus diet where the metaphysis may extend far down into the shaft Is that right, Dr McLean?

McLean That is right, but I do not think that is the question he is asking

Bloom No, but let me treat it this way Presumably there is an excess of calcium and phosphate in the animal's economy, so that for some reason or other bone is not eroded as rapidly as it might be if there were not this excess of calcium and phosphate That is, on a general bodily basis As far as the parathyroid hormone acting generally goes I think it is interesting that, in a growing animal at least the parathyroid hormone hits the metaphysis mainly, although it will also affect the cortical bone to a slight degree Why the parathyroid should affect the metaphysis in terms of your question, I do not know

As to whether, hypothetically, diminishing the amount of calcium and/or phosphorus at a point close to a spicule of bone would help resorption your guess is as good as mine

Armstrong It seems to me that in order to interpret properly the two experiments that you have just described, Dr Bloom, you need to know the actual rates of bone growth and bone elongation in the two situations It seems possible in the situation that you have drawn on the right that the rate of bone growth in the animal was greater than in the other case thus accounting for the elongation of the metaphysis

Bloom I do not think the high rate of growth would take care of it What is striking here is a diminished rate of resorption

Armstrong This, in the end, amounts to an accumulation of more skeleton

Pfeiffer What would be the explanation of bone formation in a tumor when it is growing very rapidly? In a tumor which is growing too rapidly supposedly, for its blood supply to keep up with it, the center of it is apt to form bone Where does that fit?

Follis I do not think you see that very often do you, in tumors?

Pfeiffer In mice tumors, particularly ovarian tumors you do

Follis You certainly do not in humans

Pfeiffer Bone may be seen also in very slowly growing tumors in mice

Bloom I have seen calcification but I do not recall seeing bone

Follis I do not recall ever having seen true bone metastasis in a tumor that was not a bone tumor, except in tumors that stimulate osteoblasts, such as those of the prostate and breast

Neuman I think Dr Follis might be thinking of the opposite situation in which with high calcium and low phosphorus one gets fair development of trabeculae and actual bone resorption That is the converse

Bloom That is right

Neuman That is associated with a general condition of low inorganic phosphate

There is a question as to whether a *local* reduction of the inorganic phosphate level might produce resorption in the way that a *general* reduction will

Follis Certainly you see it in other conditions besides parathormone stimulation In the absence of any parathormone stimulation I wonder how much is humoral (I mean in relation to calcium and phosphorus) and how much represents some other kind of stimulation?

I have another question I should like to ask Dr Bloom Have you used unfixed tissue that is just frozen sections of unfixed tissue?

Bloom Not frozen tissues All this work was done on frozen dried tissues

Follis You would have obtained different results I think if you had not used frozen dried but had just used frozen sections of unfixed tissue

Bloom Without letting them come in contact with water?

Follis That is right

I should also like to ask is it possible that there are other enzymes in saliva such as proteolytic enzymes (perhaps Dr Hastings can answer that) other than diastase?

Bloom I do not know

Follis You used saliva?

Bloom Yes

Follis Are there?

Hastings I do not know I think so but I do not know

Follis Are you getting any other effect using saliva that might be a proteolytic effect and not an effect on the mucopolysaccharide?

I ask that only because one can change the metachromatic staining of skin collagen by digesting it with pepsin Skin collagen of course, is ordinarily not metachromatic If one digests it with pepsin one can make it metachromatic Trypsin will not do that That is of interest I think

because it is well recognized that pepsin will digest collagen and that trypsin will not One wonders whether one is not digesting mucopolysaccharide of the collagen and perhaps liberating groups which are left behind with the diluent blue, say, making them metachromatic[7]

Bloom There are two points there I think you have to keep sharply in mind the differences between the amorphous binding substance and the collagen fiber I believe it is the amorphous binding substance that is first to go with trypsin digestion After you get rid of the amorphous binding substance, the fibers are certainly more fragile

Follis How do you regard collagen? What is your concept of how collagen is constituted?

Bloom Years ago I was interested in the question of the intercellular or extracellular origin of fibers One of the problems that had to be settled at that time was the relationship of precollagenous fibers and reticular fibers to collagenous bundles All three of them resist pancreatin digestion One of the early histochemical agents was dilute acetic acid (or dilute formic acid) Henle and others began to use it in the 1840's If you take a bit of reticular tissue, watch it under the dark field and put on a dilute organic acid 0 5% or 0 25% formic acid or acetic acid you do not see anything happen to the fibers But if collagenous bundles are exposed to dilute acetic acid you can see with the dark field that two things happen (1) The picture becomes dull It is very much like what happens when a bit of tin foil is placed on a very hot stove It loses its glistening appearance, it becomes quite dull and irregular (2) Something swells and that something I am sure is the amorphous binding substance Just what this is chemically and what its relationship is to collagen I do not know I had hoped that Keith Porter's electron micrographs would help, but so far they do not He found a beading in the very early stages of developing fibers comparable to the beading in dissected collagenous fibers—the spacing is the same

It would be very nice if it could be shown that the amorphous substance would turn into the fibers by a condensation or rearrangement What this binding substance is I do not know other than that it contains glyco or mucopolysaccharide I do not know the relation of this substance to the fibers Gersh and Catchpole were noncommittal as to the exact relationship of the amorphous substance to the fibers Is that what you mean?

Follis Yes

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[7]Follis R H, Jr The Effect of Proteolytic Enzymes and Fixation on Metachromasia of Skin Collagen, *Proc Soc Exp Biol and Med* 76 272 273 (1951)

I am not too clear myself. We talk glibly about collagen but if you try to pin someone down as I have just tried to pin you down really it is a very difficult problem to visualize.

Bloom: It is a very nicely oriented molecule and it develops normally in certain positions. If you change the mechanical forces acting on it you can change it. Chemically I cannot do more than that Dr Follis. Morphologically I could say many things.

Stetten: I should like to offer one fact and then a speculation.

In studies conducted by Dr Marjorie R Stetten on hydroxyproline we have made a survey of the occurrence of hydroxyproline. Hydroxyproline has been shown to occur in only three proteins. There is a very large amount of this amino acid in gelatin and presumably in collagen where there is every reason to believe that it occurs naturally. It is not an artifact of gelatinization. It occurs as a minor component in elastin where it may be a true ingredient or may result from contamination of elastin with small amounts of collagen—a separation which is very difficult. And it occurs in a protein from the amanita mushroom called phyalloidin but this amino acid is of the wrong optical configuration. In other word hydroxyproline as far as we know has a distribution which is almost as specific as the distribution of thyroxin in thyroglobulin. It is essentially peculiar to collagen. That is the fact I wanted to offer.

The speculation is quite wild. Thyroxin has a specific function in thyroglobulin and it may be supposed that hydroxyproline has a specific function in collagen. Yet the function of collagen as far as I am aware is simply to exhibit a high tensile strength. I do not know of any other function that it has. I should be interested in hearing any.

That then raises the question what is this peculiar hydroxyl group doing? The hydroxyl group offers points of attachment for other groupings. In the linear polymers of industry when one wants a linear fiber of high tensile strength one aims at primary valence bonds connecting the fragments together and therefore it is suggested that this hydroxyl group of hydroxyproline may be offering points of attachment for carboxyl groups either those of glutamic acid which is also abundant in collagen or of the uronic acid which is a major constituent of most of the polysaccharides which have been obtained from this source.

Follis: How about sulfate?

Stetten: I do not know. Is there much?

Follis: Chondroitin sulfuric acid? I think Karl Meyer[8] has postulated

hasn t he, that it is a simple salt linkage between the protein of cartilage and chondroitin sulfuric acid?

Stetten I am not talking of the salt linkage, I am talking of the ester linkage There is sulfate in cartilage I do not know whether there is any appreciable amount of it in fibrous connective tissue

Follis There is chondroitin sulfuric acid in skin collagen

Johnson While there may be chondroitin sulfuric acid in skin, I did not understand that it was a part of the collagen per se In the leather industry where there is a vast literature on the collagen problem it seems that they have gotten almost all the substances out and are dealing with pure fibers pretty much There is an excellent review of that

Follis What I am talking about is undenatured collagen The leather industry is concerned with denatured collagen most likely If one does not do such drastic procedures as tanning with various chemicals, one can isolate the chondroitin sulfuric acid and hyaluronic acid from skin collagen

Hendricks I just have to get out of my field all the time I do not remember its formula immediately, but isn t the function of proline to reverse the angle of bend in a polymer? I think there is an extensive literature on the subject, and it is contended that one of the things that distinguishes the beta keratins from the alpha keratins is the presence of proline to permit the molecule to bend back

Follis You will have to ask Dr Stetten about that point

Stetten I am not familiar with the literature to which you refer We have repeatedly tried to recover hydroxyproline from such tissues as liver, which is pure in collagen, and we have been unable to get it

Hastings Wouldn't hydroxylysine act in the same way?

Stetten Hydroxylysine has also been demonstrated in gelatin

Hastings Yes, it is higher in gelatin than in other proteins

Robinson In talking about the bone that you are staining with silver nitrate, you are talking about fragments or pieces of whole bone, aren't you? I mean, what proof is there that it particularly stains the hydroxy apatite crystal and not the organic matrix?

Bloom The same methods applied to uncalcified bone do not stain, as in rickets, for instance

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[8]Meyer, K Palmer J W and Smyth E M On Glycoproteins V Protein Complexes of Chondroitinsulfuric Acid *J Biol Chem* 119 501 (1937)

Robinson What about deproteinized bone? Does that stain with the silver nitrate?

Bloom Yes

Robinson The reason I ask that question is that, in order to get a sharp focus in certain specimens with the electron microscope, we put silver nitrate in with deproteinized bone and the silver nitrate forms small globules in the field which are very dense black and have a sharp border It is possible to focus on them They lie on the plane of the screen so that the hydroxy apatite units that are lying about can be brought into sharp focus that way But these globules do not appear to be directly connected to the hydroxy apatite crystals They are just spread out through the field They show no particular relationship to them One would not even think they were first cousins

Armstrong Have you a slide that you can show?

Robinson I do not have a slide of that particular specimen This specimen was taken from a sclerosing osteoma of the tibia It shows rather large hydroxy apatite units and you can see the silver nitrate in small globules through the field and scattered about without being attached

Johnson Dr Bloom I wonder whether it would be feasible to explore the possibility of tying together the experiments with the salt you have been carrying out particularly as seen in the metaphysis and the work on the radioactive carbonate that you previously did (I do not quite know right off hand what the technical procedure would be) and possibly find out a little bit more about the exact nature of this change in the bone salt that you are seeing around the osteocytes in the metaphyseal area Would it be technically possible for instance to distinguish carbonate from phosphate on the basis of weak acid hydrolysis and demonstrate it under one set of circumstances and not the other?

That I think is the kind of thing implicit in the question originally directed to Dr Hendricks

Bloom I do not think so Dr Johnson I do not think you could make this distinction on individual bone spicules either by dissecting them out or by using autoradiographs There is a bare possibility that you might be able to do it if you used a large number of animals

Johnson I was not thinking of using a radioactive tracer again but that the two types of data or study may be related to each other

Armstrong I am always amazed at how we can start out on one subject and find that the discussion leads into many bypaths I do not think that anyone could have anticipated the direction in which this discussion has gone

SOME HISTOCHEMICAL OBSERVATIONS ON THE DEVELOPMENT OF MEMBRANE BONE*

GERRIT BEVELANDER and P L JOHNSON

From the Department of Histology College of Dentistry and the Graduate School of Arts and Science New York University New York N Y

Armstrong Dr Bevelander would you please go ahead

Bevelander I should like to make a few remarks on the investigation that we have been making for some time with particular reference to certain features in regard to the genesis of membrane bone A great deal of work as you know, has been done on the study of the development of bone, but for some reason membrane bone has been to a large degree neglected in many studies We think that our investigations of the development of membrane bone are of particular interest with reference to studies made of endochondral bone and endochondral ossification

We examined the development of bone in pig embryos which were prepared for the most part, by the freeze dried technic The presentation to be detailed in the following paragraphs is confined to observations on the histogenesis of membrane bone The topics to be dealt with concern the incidence localization and interrelationships of the following (1) alkaline phosphatase, (2) mucopolysaccharide (3) glycogen and (4) mineral salts with special reference to cellular matrix and calcification areas

Material and Methods

Mandibular regions of young pig embryos were fixed according to the Altmann Gersh freeze dried technic for subsequent histochemical treatment while other specimens following 95% alcohol fixation were decalcified by the Lorch method Modifications of Gomori's method for demonstrating phosphatase were utilized Such a variation which we developed wherein magnesium chloride substituted for calcium nitrate in the incubating medium proved most successful in localizing phosphatase in the bone matrix

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*These studies were supported in part by a grant from The National Institute for Dental Research of The National Institutes of Health U S Public Health Service

[1a] Gomori G Microtechnical Demonstration of Phosphatase in Tissue Section *Proc Soc Exp Biol and Med* 42 23 26 (1939)

For the identification of glycogen and polysaccharide the method of Hotchkiss[11] was employed and supplemented by the Gage iodine technic By means of salivary digestion the glycogen was separated from the mucopolysaccharide The mineral deposits were studied exclusively in unde calcified material, fixed according to the freeze dried method and subsequently treated by a modification of the von Kossa stain and counterstained with light green

Results

1 Alkaline Phosphatase

Figure 1 is a section through the head of the pig embryo that gives you an idea of the part of the embryo which we studied We studied the region of the mandible in the developing head This section illustrates the distribution of alkaline phosphatase in the embryonic head We were particularly interested in the regions that were about to undergo calcification and subsequently did undergo calcification Among other things, this preparation shows the fact that alkaline phosphatase is not only localized in those presumptive areas of calcification but also in a great number of other tissues for example, in the tongue and the vascular elements of the brain, and so on

Follis Do the fibers in the tongue show activity, that is, the fibers themselves?

Bevelander The muscle fibers?

Follis Yes

Bevelander I believe so Dr Follis You cannot tell from this figure, of course

The phosphatase in bone has been studied extensively by a great number of people, and talked about previously at this Conference, and many points in connection with its localization and possible function have been brought out In our studies we were not very well satisfied with the methods available for localizing phosphatase in tissues, particularly in calcified tissue Most of you realize there are a number of inherent technical difficulties in preparing sections of calcified material

When one treats a section to visualize phosphatase by the method described by Gomori the resulting precipitate in the bone matrix is ex

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[11]Hotchkiss R D , A Microchemical Reaction Resulting in the Staining of Polysaccharide Structures in Fixed Tissue Preparations *Arch Biochem* 16 131 141 (1948)

tremely black the periosteum fibers and surrounding tissue are very dense and it is very difficult to tell precisely what one is looking at under the microscope A year or two ago a modification was developed both here and in England in which decalcification preceded treatment of the tissues which were subsequently reactivated By this method it was claimed that fairly good representations of localization of phosphatase could be obtained

We have tried these methods both the methods advocated by Lorch in England and the methods advocated by the Harvard group and we obtained preparations such as is shown in Figure 1 This illustration does not quite do the method justice but I think you can see that you get a rather washed out appearance of all of the tissues and cells

In an attempt to obtain more precise localization and visualization of alkaline phosphatase in developing membrane bone than has heretofore been reported by means of Gomori s method or subsequent modifications of this method involving either undecalcified tissue or decalcification with subsequent reactivation of the enzyme we experimented with freeze-dried material followed by incubating mixtures containing a number of divalent cations We found that when we substituted magnesium in the usual incubating mediums in place of calcium we got a rather unusual picture in regard to localization of phosphatase in bone During the course of these experiments we[12] were able to observe in those specimens incubated in mixtures containing magnesium ion substituted in place of calcium ion the following points

(1) Considerable amounts of phosphatase were observed in the usual complex of tissues and cells such as periosteum matrix of young spicules osteoblasts and osteocytes

(2) Rather comprehensive areas of the developing mandible anlage (Figure 2) in which mineralization had occurred in one region but not in another revealed (a) definite indications of the presence of phosphatase in the cellular components and matrix of the uncalcified regions and (b) a gradual decrease and eventual loss of phosphatase in the calcified areas in which mineralization was taking place with the exception of the osteoblasts osteocytes and osteoid in which it was still prevalent

In regard to this latter observation it has previously been held[13] that the developing matrix of bone was lacking in phosphatase More recent

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[12]Bevelander G and Johnson P H A Histochemical Study of the Development of Membrane Bone *Anat Rec* 108 1 2? (1951)

[13]Freeman S and McLean F C Experimental Rickets *Arch Path* 37 387-408 (1941)



Fig 2 Section of Head of Pig Embryo

Higher magnification of the developing bone represented in Figure 1 (a) showing (a) the presence of phosphatase in the uncalcified portion of the matrix and (b) the distribution of phosphatase in the calcified areas Reprinted by permission from Bevelander G and Johnson P L A Histochemical Study of the Development of Membrane Bone *Anat Rec* 108 1 22 (1950)

studies[14 15 16] however substantiate these observations that phosphatase is localized not only in cellular components but also in the bone matrix before calcification occurs Of interest also in this connection is the observation that our phosphatase preparations show a positive reaction for this enzyme on the osteoid border of the developing spicule This result is at variance with the findings of previous investigators

The section in Figure 1 which was treated to show the localization of phosphatase in the growing spicule reveals that in the upper portion of this tissue which is uncalcified the enzyme appears to be abundant in the matrix the lower portion of the bone which has undergone partial calcification is lacking in phosphatase Examination of this same tissue at a higher magnification (Figure 2) shows in addition to the features already pointed out that the tissues surrounding the matrix viz, the pereosteum is rich in phosphatase and the enzyme is also abundant in osteoblasts and osteocytes We have demonstrated in such preparations that phosphatase appears fairly abundant in the uncalcified matrix and that it gradually disappears as calcification proceeds

Follis It is certainly present in the fibers of the osteocytes isn t it?

Bezclander Oh yes

Follis That has been recognized for some time

Bezclander As to the actual presence in the matrix there have been two papers I think in the last two years one by Dr Lorch in England who observed patches on the periphery of the young bones of kittens and I believe Wang who studied the development of Ancon sheep Although he did not give detailed data on phosphatase he made the statment that the presence of phosphatase preceded calcification

2 MUCOPOLYSACCHARIDE

Another constituent in developing bone which we examined was mucopolysaccharide which was studied by the Hotchkiss method as has been outlined by Dr Bloom this morning Mucopolysaccharide was ascertained in the several developmental stages of bone formation Our preparations

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[14]Chang T K Calcification in Fetuses of Normal and Ancon Sheep *Anat Rec* 105 723 735 (1949)

[15]Lorch I J The Localization of Alkaline Phosphatase in Mammalian Bones *Quart J Microscop Sci* 88 376-383 (1947)

[16]Lorch I J The Distribution of Alkaline Phosphatase in the Skull of the Developing Trout *Quart J Microscop Sci* 90 183 207 (1949)



Fig 3 Yo ng Sp cule of Develop ng Membrane Bone

a) Young sp cu e devo d of m n al sal s how ng gl gen n e u ou d ng

9 0)

indicate that this substance is confined at first to osteogenic fibers and later comes to be more generally distributed throughout osteoid tissue This polysaccharide appears in its general distribution to become incorporated into the cementing substance of the entire matrix of older spicules prior to and during mineralization and incremental growth showing a much more intense staining reaction in more mature than in the young spicules

In a freeze dried preparation of a very young spicule of developing membrane bone one sees (Figure 3) that the osteogenic fibers which are becoming or about to become incorporated into the young spicule stain fairly well by this means which indicates to us the presence of a mucopolysaccharide in the osteogenic fibers As the fibers become incorporated into the growing spicule the visibility gets to be less discrete as the spicule becomes older In addition to the mucopolysaccharide indicated in these areas of bone matrix we also observed considerable amounts of glycogen

3 Glycogen

Our studies revealed considerable amounts of glycogen associated with areas of membrane bone formation At first it is widely distributed in the undifferentiated mesenchymal tissue With the incidence of a definite spicule anlage wherein osteoblasts appear to be differentiated these cells momentarily lose their glycogen This condition persists until just prior to mineralization when both osteoblasts and osteocytes attain a maximum in glycogen content Except for a brief phase in development glycogen is abundantly present in periosteum osteoblasts and osteocytes throughout the stages of development and calcification

In the uncalcified portion of the spicule of Figure 3 the mucopolysaccharide is relatively much less intense in its staining quality than it is in the portion which begins to undergo calcification This particular section was stained for both glycogen and mucopolysaccharide Glycogen is abundant in the surrounding periosteal tissue and in the cells osteoblasts and osteocytes In a considerably older piece of bone the trabeculae again show the localization of both glycogen and mucopolysaccharides This is not present as a discrete pattern but as a profuse staining reaction and one cannot be sure that it is present in both fiber and matrix or in just one or the other

In addition to the intense staining reactions which we obtained for polysaccharide in fairly well calcified trabeculae we also observed intense localization of glycogen in both the osteoblasts and in the osteocytes

In regard to the localization of glycogen in developing membrane bone we have observed one interesting feature namely that glycogen appeared

to be present in the cells which were a little more differentiated than fibroblasts but could not quite be called osteoblasts Then for a time the glycogen appeared to disappear from the cells as they lined up along the spicule which was beginning to undergo organization

It appears that as soon as calcification of the spicule begins glycogen is again present in the osteoblasts and in the osteocytes and during all the period that we studied the differentiation of bone in which it had undergone calcification there seemed to be abundant glycogen in both osteoblasts and osteocytes

4 Mineralization

Mineralization as shown by modification of the von Kossa method reveals minute isolated calcareous deposits in and among the well established osteogenic fibers and occurs subsequent to phosphatase deposition In more advanced stages of spicule formation these isolated deposits coalesce and fuse entrapping osteocytes

The preparations in Figure 4 are from sections adjacent to those previously shown and were fixed by the freeze dried method They were treated to show localization of mineral salts by Bloom and Gersh s modification of the von Kossa method In the terminal end of a very young spicule we observe mineral deposits in the matrix proper as one might expect and also among the delicate osteogenic fibers which eventually become incorporated into the developing spicule

5 Summary

Thus the pattern of interrelationships of these substances occurring in developing membrane bone appears to coincide with the pattern of these same substances which has been reported in connection with the development of long bones As Dr Bloom has remarked it is perhaps unwise to say very much about what these things mean in terms of physiology or growth of the bone I think there is one thing however we might conclude from these studies and that is the constituents that we examined in membrane bone appear to be very similar and to follow a very similar pattern in origin and distribution as compared to cartilage replacement ossification

Conference Discussion

Armstrong Thank you very much

Ishing Would you review one point just briefly? As I have it here we saw one figure in which the mucopolysaccharide was found in very delicate fibers which were being incorporated into bone and became of lessened



Fig 4 Developing Membrane Bone

(a) and (b) Different areas of developing bone showing localization of mineral salts Reprinted by permission from Bevelander G and Johnson, P L A Histochemical Study of the Development of Membrane Bone *Anat Rec* 108 1 22 (1950)

visibility as we approached the mass that was well incorporated or well established

Bevelander That is right

Asling Yet subsequently we saw an old spicule with a very markedly positive reaction

Bevelander That is right but we could not discriminate the fibers any more in that section

Asling I am afraid I do not yet understand Why did we get a diminished visibility? Why did we see a superstrong reaction in an old well established trabecula?

Bevelander We got a more intense staining reaction in the older trabeculae But in the trabeculae when the spicule becomes calcified one is not able to discriminate fibers in the matrix One cannot tell the difference between the fibers and the cementing substance any more

Gutman Dr Bevelander when you say that the calcification processes in membrane bone resemble those that have been suggested in endochondral calcification could you tell me more definitely to what you are referring?

Bevelander I am referring to the presence of phosphatase in the periosteum in the osteoblasts in the osteocytes and according to the latest studies on the topic in organic matrix I am not sure about the polysaccharide—whether it is strictly comparable or not

Follis Certainly there is an accentuation of the fibrillar structure of cartilage when one stains it with modifications of the Hotchkiss technic Wouldn t you agree Dr Bloom that the fibers one sees in the epiphyseal cartilage certainly stain much more intensely than the interfibrillar substance whatever that may be?

Bloom I do not know I am not too sure Dr Follis

Follis Certainly they are more intensely metachromatic

Bloom I have a very hard time with fibers and cartilage anyway

Follis If one does something that will weaken the cartilage such as to produce scurvy so that mechanically the cartilage must be stretched one can bring out the fibrillar pattern much more readily and certainly when one stains it with either of these technics I think one is impressed by the more intense staining of the fibrillary structure in relation to as I said the interfibrillary material whatever that is

Bloom I have seen such preparations but I am not sure that I would

identify all of those fibrils with the fibrils that have been demonstrated by Bielschowsky It is pretty hard to identify them

Follis Of course there is continuity of the fibers from the marrow or at least from the connective tissue cells or the osteoblasts or the marrow as they go into the bone and there their identity is lost because of the calcification They certainly are more intensely stained also You see that very beautifully in rickets where you don t have to worry about the presence of inorganic material masking the staining

Of course those are the fibers that give a positive silver reaction are they not?

Bloom Yes

Follis As they get in and apparently are more compact and perhaps more organized the silver reaction disappears?

Bloom Partially

Follis Yes

Bloom I should like to add only one sentence to Dr Bevelander s comments As this bone gets older and denser it will lose the ability to stain with the Hotchkiss method

Follis How about it if you decalcify it?

Bloom As soon as you add any acid the staining is upset

Follis Do you think that that is because as was brought out in the first meeting in 1949 by Dr Levine [17] you have treated it with acid? Of course you do treat it with acid in performing this histochemistry Or do you think that it is due to the removal of the organic material?

Bloom I think it is both In addition there is diffusion

Asling There is a technical possibility that has offered itself—perhaps some of you have tried it—using chelating agents Versene is one of the commercial agents and decalcification of the bone is done very nicely in a completely neutral medium with this

Follis Or even in an alkaline medium

Asling I have been dissatisfied with conventional histologic stains with it but it might offer some assistance in these histochemical procedures

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[17] Levine M D Rubin P S Follis R H Jr and Howard J E Histochemical Studies on Calcinosis Universalis with Respect to the Possible Relationship Between Normal and Pathological Calcification *Trans Macy Conference on Metabolic Interrelations* 1 41 (1949)

Follis It certainly takes the inorganic materials out I can confirm that

Asling The metachromasia is distinctly disappointing

Bloom Dr Engel at the University of Illinois has made a very interesting observation concerning the mobilization of mucoproteins by parathyroid hormone He found that in rats the amount of mucoproteins in the plasma was practically tripled in 48 to 96 hours after the injection of parathyroid hormone

Follis Has that been published?

Bloom It will be in the *Federation Proceedings* for 1951 I have his abstract

STUDIES ON THE EFFECT OF ALTERATION IN THE CONCENTRATION OF CALCIUM IN CIRCULATING FLUIDS ON THE MOBILIZATION OF CALCIUM[18]

A BAIRD HASTINGS

From the Department of Biological Chemistry
Harvard Medical School Boston Mass

Armstrong Dr Hastings by chance do you have a slide in your pocket that you can show us now?

Hastings I wrote you that I wasn t going to speak, I was just going to listen this time

Armstrong Oh but you have to

Hastings What I have to talk about is only tangential to this business It is physiological rather than histologic

I was afraid you would do this I do not have any hot slide just off the griddle as I did last year The only reason that I have something to say today is that my secretary and I were trying to organize our slides which had gotten into pretty bad shape over the last twenty years and I ran into these data The experiments are just about twenty years old this month They have never been published so I thought you might like to hear about them

The studies have to do with a crazy idea that Dr Huggins and I had a good many years ago We thought we would produce tetany in dogs by a new and unconventional way We would remove all the calcium that we could get out of the serum from a large batch of blood from one donor dog Then we would put that plasma back with its cells and then transfuse repeatedly a small recipient dog and throw him into tetany Well these investigations are about that but there is something in them that may interest you interest Dr Follis at any rate It pertains to his question of the mobilization of calcium by effecting the concentration of the calcium *in the circulating fluids*

The way we removed the calcium was by shaking the serum with lead phosphate which is very insoluble We were studying adsorption of cal cium *from the serum* As *one* shakes serum with more and more phos phate one finds after centrifuging out the lead phosphate decreasing

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[18]These studies were done in collaborat on with Dr Charles C Huggins now of Chi cago Ill nois

amounts of calcium in the serum It may amount to only 0 1 millimole whereas we started at about 2 6 So we have taken out over 90 per cent of the calcium from the serum

We used such serum that had only between 5 and 10 per cent as its initial calcium concentration and then it was put back with the cells and that blood was then injected into the dog

Dr Huggins was on one side removing about half the dog s volume as quickly as he could from one femoral artery and I was on the other side immediately putting back an equal volume of blood

We did this eight times in the course of two and a fraction hours and we analyzed the serum of the blood that we withdrew at each point We thus made a 50 per cent transfusion every twenty minutes In this experiment the plasma calcium was not greatly decreased

So we tightened up our procedure and managed to give a transfusion every ten minutes or actually we were working continuously If the calcium that we were removing came entirely from the plasma and the other extracellular fluids of the animal and none came back in from bones we would have expected to have reached a very low plasma calcium value rapidly However this was not the case We took out about four times as much calcium as could have been present in the total extracellular fluids Still the plasma calcium remained relatively high

As you see by giving these transfusions every ten minutes we finally managed to get the plasma calcium down below the 7 mg level but not nearly as low as we expected

We never really saw tetany We looked very hard We would get quivering of localized groups of muscles but it was not tetany in the usual sense of the word

We were able to keep the plasma calcium down as you see but it did not get anywhere near the level that it would have gone if calcium were not coming back in at a terrific rate

Follis May I ask were these animals starved Dr Hastings?

Hastings I do not know Twenty years is a long time for me to make any positive statement about their nutritional state As far as I know it did not occur to us to starve them

We performed another experiment of the same sort except that this time we wondered whether the calcium would come back after we stopped At the twenty five minute level it was back up above the tetany level and it was back to two millimoles per 100 cc an hour after we stopped

At the same time, we got some cerebrospinal fluid and it was a perfec normal figure for calcium

This was an animal that had been parathyroidectomized about six hou before we started the experiment

In still another study the animal had been parathyroidectomized thr hours before the experiment He wasn t in tetany but we got down w below the tetany level very much more promptly than with normal anima The cerebrospinal fluid calcium was a little low but interestingly enoug even higher than the serum calcium which of course is about half bour with protein and half free So there was a big discrepancy in calcium ic concentration between that in the cerebrospinal fluid and the plasma

Robinson You thought that was just a matter of time though didn you?

Hastings Oh yes

The only reason I thought all these studies might be of interest today that they show the great rapidity with which calcium can be mobilized

I shall be very glad to answer any questions

Conference Discussion

Armstrong Were your animals anesthetized during these experiment on withdrawal transfusions?

Hastings Only locally for the purpose of getting to the blood vessel

Robinson Did you look at any bones after this treatment?

Hastings No this is all the story there is

Armstrong Well this certainly is a demonstration of the rapid equi libration of change between the extracellular fluid in the skeleton

Hastings Why it is terrific When you can take out many fold the amount of circulating calcium you have present and cannot even begin to approach the concentrations that you would expect to find based on what you are putting in with only ten minute intervals it means that we can mobilize calcium very rapidly My moral, for Dr Follis question, is that the low concentration of the calcium is in itself a stimulus to mobilization

Armstrong Along the lines of the observations you have made, Dr Hastings Dr Singer and I have studied the dilution of injected radioactive calcium We see in our experiments that in 10 minutes the dilution of the radiocalcium is greater than the volume of the entire extracellular fluid This fact indicates a rapid exchange of calcium between the interstitial

fluid and the skeleton In the experiments using radiosodium, within 10 minutes after the injection the dilution of the isotope has reached a volume equivalent to that of the extracellular fluid.

McLean You did not do phosphate analysis?

Hastings Franklin, we did, but I don't think we ever got them plotted

McLean The lead phosphate will also take the serum phosphate down to about the same degree

Hastings No, you are forgetting It takes it down but it does not take it down nearly as far

McLean Not nearly as far?

Hastings No, it takes out 50 to 60 per cent under the same conditions

McLean But under these conditions, you are probably also mobilizing phosphate

Hastings I should think so You have to

McLean Of course, you have more sources for phosphate, too There are more places phosphate could come from

Hastings Yes, but that would be in the direction, from a physical-chemical point of view, of cutting down the amount of calcium you get, the more phosphate you got

McLean It seems to me the interesting thing about this experiment is that calcium can be mobilized so rapidly in the absence of the parathyroid gland

Hastings Yes

McLean If you had not done the parathyroidectomies, one would have said the parathyroids were hyperactive

Howard How long did you parathyroidectomize that animal before you did it?

Hastings Thirty hours

Howard Our experience is that most of those dogs are in tetany then

McLean This was almost in tetany

Howard Without taking any calcium out of the blood at all, most dogs are in tetany by that time

Hastings You see them in tetany and you see them not in tetany.

Howard That's right but you see enough in tetany to make you think there is something screwy about it

Hendricks One of the animals was parathyroidectomized six hours before?

Hastings Yes

Howard We intended to do a similar experiment to yours with dialyzing blood in a calcium free solution but had not gotten around to it before we started in the opposite direction We gave large amounts of calcium intravenously over a period of three hours bringing the plasma up to a level of where it would be in a parathyroid tumor actually We did it in normals and a patient who had had a parathyroid tumor after the removal of the parathyroid tumor The serum calcium remained the same as it had been before the tumor was removed We could account in the urine in forty eight hours for about a half of the calcium we put in and yet the serum calcium is back to normal in a few hours This indicates that the compartment for calcium is elastic in both directions just as it is with the injection of potassium salt

Shorr May I ask whether you equilibrated the blood with oxygen and CO? Could there have been any pH changes?

Hastings No I wouldn't miss that We did pH and CO once in a while They were all right

Armstrong I think that these observations teach us again that we as rather highly developed animals have simply taken advantage of the bones to produce rigidity of the body and that the more primitive function of the skeleton is something else The skeleton is a compartment which allows exchanges between itself and the active solutions of the body In this way the skeleton serves to aid the processes which maintain the normal concentration of several electrolytes in the extracellular fluids It is only accidental from an evolutionary standpoint that the bones furnish rigidity to the body

Hastings It makes me also think some of the old solubility product laws are pretty good The stored calcium will still respond to lowering calcium ion concentration

that is a low phosphorus diet and no D at all in a matter of six hours the animal is in tetany when you starve him So that there is something about the bone which in this one set of circumstances can support the calcium in the serum but under other circumstances cannot do so

A clinical observation that has puzzled us for a long time is that one person with steatorrhea in a period of diarrhea (because they have cyclic episodes of it) will go into tetany in a matter of a few days of that diarrhea whereas another fellow will have bones so rare you can practically see through them through the x ray and will have normal calcium and no tetany at all There must be something very different about the state of the bones as to when they can support serum calcium and when they cannot

Follis Of course under such a circumstance you begin to heal your rickets

Armstrong That is the point I was going to make

Follis It may be that the calcium takes the phosphorus into the bone and the rickets begins to heal That might account for the lowering of calcium might it not?

Copp What type of animal organism showed this vitamin D effect?

Howard You mean without the D it can t support?

Copp Yes

Howard That was the rat

Copp We have had great difficulty in getting tetany in the rat on low phosphorus diets

Howard I should not have said tetany I should have said tetanic level because I do not remember in those animals whether they actually twitched But they had very marked low calcium

McLean There is also an old observation in the literature I think it is in Bengt Hamilton s work [19] He amputated one leg of a normal rat and determined the calcium in the bone in that leg Then he put the animal on a rachitogenic diet and allowed it to remain on that diet for some weeks until the rat had developed low phosphorus rickets in the other leg Then he analyzed the other leg for calcium and found that the animal had not lost calcium from that leg In other words his testimony in so far as it

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[19] Hamilton B and Hegelman, W J Jr The Changes in Total Calcium Content of the Bones During the Development of Rickets *J Nutr* 15 177 (1938)

goes is to the effect that it is very difficult to mobilize the calcium already deposited in a rachitic animal, since a rachitic animal does not lose a great deal of mineral from the already calcified bones

Howard No, but the bone in that leg had grown, had it not?

McLean It had grown

Howard Therefore bone for bone, it was a lot bigger, therefore, mass for mass there was a lot less lime in it

McLean Oh much less But what you would expect if calcium is so easily mobilized is that a rachitic animal would lose all of its calcium from its bones or at least a considerable proportion of it

Howard Was that low calcium rickets or low phosphorus rickets?

McLean It was low phosphorus rickets

Howard So there was adequate calcium in the diet to keep up the serum level?

McLean Yes

Follis You can of course, throw rachitic animals into tetany by doing what you and Dr Urist did in your study on healing by injecting phosphate into the peritoneum You probably have killed animals by giving them too much just as I have They do have tetany and they twitch

McLean Oh, yes

Hastings You can produce tetany in animals by feeding them enough phosphate by mouth Salvesen, McIntosh and I [20] did that years ago

Pfeiffer Is there any evidence in the literature as to what part of the bone or any location, this calcium comes from?

Hastings You showed it didn t you, Dr Bloom?

Bloom I wouldn t say so except that in growing animals, the most labile part of the bone is the growing part

Hastings Was there growing bone all the time?

Bloom Yes

Pfeiffer And that means before it is reorganized wouldn't you say?

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[20] Salvesen H A Hastings A B and McIntosh J F Blood Changes and Clinical Symptoms Following Oral Administration of Phosphates, *J Biol Chem* 60 311 (1924)

Follis Wouldn t you say the calcified matrix is probably the most labile?

Bloom No What I mean by labile is that it is harder to get calcium out of the dense bone of the cortex for instance

Follis It is being destroyed

Hastings It is certainly where there is the best blood supply I should say

Follis That is the area

Hastings This is terrific, that gap from a tenth of a millimole per liter and two millimoles per liter in ten minutes

McLean This comes back to Dr Bloom s diagrams and to Joe Aub s demonstration[21] that animals store calcium in the trabeculae of the spongiosa When animals are deprived of calcium they mobilize it from the spongiosa When they have an excess of calcium as has recently been published from our laboratory the spongiosa is not resorbed and grows to a great length[22] One can reproduce in rats the same picture one produces with estrogens simply by giving enough calcium so that the animal does not need to resorb the trabeculae of bone

I think there is no doubt that in growing animals—we are talking now only about animals that are still producing spongy bone—the easiest place from which calcium may be mobilized is the trabeculae of the spongiosa Of course in an adult rat or an adult animal of any kind the skeletal system is much more stable

Pfeiffer Were these adult dogs?

McLean These probably were

Hastings No they were mostly puppies They were small dogs in all cases because we had to get enough blood to make so many transfusions

Pfeiffer Could you use old dogs as well as young dogs?

Follis You would probably get less I should think a young growing animal would give you more of a response than an adult dog that is it would come back quicker

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[21] Bauer W Aub J C and Albright F Calcium and Phosphorus Metabolism V Study of the Bone Trabeculae as a Readily Available Reserve Supply of Calcium *J Exper Med* 49 145 (1929)

[22] Carttar M S McLean, F C and Urist M R. The Effect of the Calcium and Phosphorus Content of the Diet Upon the Formation and Structure of Bone *Am J Path* 26 307 (1950)

Hastings We regarded these observations as a failure because we did not succeed in producing tetany in a new way

Follis Certainly that is true in children too

Urist There is another way in which one can mobilize calcium and wipe out the spongiosa of the long bones all over the skeleton A normal animal placed on a starvation regimen will show extensive resorption of bone tissue In a matter of a week the spongiosa will be absorbed all over the skeleton producing a condition described in the post war European liter ature as hunger osteopathy

Follis Oh yes

Urist And like in all the experiments just mentioned the process is more rapid and more complete in the young than the adult The fixation of the bone salt seems to be determined by the rate of cellular activity and growth

The cellular activity in the bone tissue appears to dispense calcium from the spongiosa which Dr Aub aptly described as the readily available bone store

Hodge We certainly do not have to assume that one is actually dis solving bone spicules or crystals to account for the kind of phenomenon Dr Hastings just described

Armstrong What did you say?

Hodge I say you don t have to require the dissolution of bone crystals and spicules to account for the kind of phenomenon that he is talking about

Armstrong You have to require that the calcium comes from some place

Hodge You have to mobilize calcium from some place, we do not have to tie this in with the idea of the removal of whole units of bone either inorganic units or structural units such as a spicule or a trabecula

Hastings You would have to take it away from the organic calcium salt if you will of the mucoproteins or the collagen or however it is found in connective tissue That is about the only source of it

McLean Are you going back to Koelliker?[23]

Robinson I think Dr Hodge stated it correctly But I think there is an element of confusion Dr Hodge states that you do not have to remove

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[23] Koelliker A *Die Normale Resorption des Knochengewebes und ihre Bedeutung fur die Entstehung der Typischen Knochenformen* Liepzig F C Vogel (1873)

fully organized units of bone at the gross or light microscope level of organization for instance trabeculae to quickly liberate large numbers of calcium ions It is difficult to see how whole biological units could be destroyed by cellular activity so rapidly

However if one considers that the hydroxyapatite bone crystals are very minute and if one then calculates their total surface area it becomes clear that body fluids are in contact with several acres of crystalline surface in any one animal It is reasonable to assume that calcium atoms of these crystals are in equilibrium with the ions of the calcium in the cement substance portion of bone matrix This matrix at least at the periphery of trabeculae and on the periphery of Haversian canals is in equilibrium with other tissue fluids including the blood plasma (This equilibrium only to a degree may be controlled by the osteocyte of the area) If one removes calcium from the blood plasma this loss is quickly transmitted as a demand on the bone matrix in which the hydroxyapatite crystals lie and into which the calcium atoms of these crystals are partly dissociated as calcium ions Calcium removed from the matrix causes a deficit in the matrix To correct this deficit an enormous number of the hydroxyapatite crystals can each contribute a few calcium ions Unless some third factor simultaneously comes into play (such as a change in the dissociation constant of calcium in the cement substance due to changing osteocytic activity) the equilibration of calcium in the blood plasma could occur very rapidly without removal of whole units of bone

Armstrong That is quite clear

Robinson Of course if this process continues long enough removal of whole units of bone does occur By then local osteocyte morphology and function has apparently changed The fact that the trabeculae are the gross anatomical units that disappear first would indicate that they are the units that have the best blood supply and therefore the greater rate of equilibrium between matrix and blood plasma

Pfeiffer That still does not mean that calcium is removed without the removal of total bone does it Dr Bloom? I thought that bone has to be resorbed to free calcium from it

Bloom That is right I would call your attention to the fact that in a laying chicken more than half of its accessory bone is torn down within six hours It puts five grams of calcium carbonate into the eggshell in that period and more than half of this amount comes from bone

McLean And that is enormously greater than the amount carried in the blood of the chicken?

Bloom Yes

McLean Even though the chicken does carry excess calcium in the blood the amount carried in the body fluids is insignificant compared with the amount that goes into the shell of the egg which is calcified within a few hours

Howard Did you say Dr McLean that if you gave enough estrogen to fill up the whole marrow cavity of a mouse—because that is the one you could do it with and not a rat—and put that animal with the estrogen on a very high calcium diet that the marrow cavity was spared that problem, is that right that is it didn t fill up? What was the remark you made about estrogen treated animals? I did not get it

McLean Estrogen rats I spoke about estrogen treated rats in which the trabeculae simply fail to resorb Is that the remark you meant?

Howard Fail to resorb under estrogen?

McLean In rats

Urist You said that a high calcium diet inhibits bone resorption Will you elaborate on that statement?

McLean A high calcium diet produces the same picture as estrogens in rats but only in rats

Urist Yes to a limited degree

McLean The first person to demonstrate that this is the mechanism of the action of estrogen in rats was Dr Follis Dr Follis showed some years ago that the effect of estrogen in rats is not to produce any new bone but to inhibit resorption of the trabeculae Isn t that true Dr Follis?

Follis Yes

Pfeiffer Actually isn t it true that the reason bone is formed under estrogen treatment is to deposit the calcium and phosphorus?

McLean Not in rats In rats there is no evidence of any increased deposition of bone All the evidence in rats is of decreased resorption

Pfeiffer Well yes I am thinking of mice You are talking about rats

McLean I am talking about rats

Asling Didn t you report Dr McLean some extremely high estrogens with slight activation of endosteum a couple of years ago

McLean Not in rats

Follis Has anybody to your knowledge studied estrogen on low calcium intakes?

McLean Yes Dr Bloom has done that with birds

Follis I mean in rats

Urist It has been attempted on rats Norman and Mittler[24] injected estrogen and found no specific effect upon animals on rachitic diets The experiment is difficult to do in rats because they will not grow on a low calcium diet

We[25] examined the bones of estrogen treated mice on calcium deficient diets and found the usual picture of endosteal bone formation but a great deal of it was uncalcified osteoid tissue The action of estrogen on the bones was not dependent upon the supply of calcium in the diet

McLean That shows up beautifully in the birds Dr Bloom has produced birds with all of the accessory bone in the marrow cavities and in a rachitic state—almost pure osteoid

Bloom When a laying chicken is put on a calcium deficient diet the bird takes calcium from the skeleton not from the accessory bone which is now uncalcified The skeletal bones become very fragile as a result of resorption The process is complicated by the fact that the birds do not want to lay under such conditions But in the spring if the diet is not too deficient they will lay occasional eggs Their bones become so fragile that I have broken a number of bones just taking a chicken out of the cage

Hastings Have they shells on their eggs?

Bloom Yes, sometimes well calcified sometimes poorly calcified

Pfeiffer This brings me back to a question I was thinking about during the discussion following Dr Bloom s paper this morning in regard to the difference between mice and rats in relation to the amount of bone formation particularly with all of the types of transplants other than para thyroid Is that tied up with the same mechanism as is the difference between mice and rats in relation to estrogen stimulation of bone or how would you explain that?

Bloom I think part of it is due to the fact that the mouse is practically an adult by the time we work on it

Pfeiffer What were the ages?

Bloom The rats weighed 40 to 70 grams the mice weighed 25 grams

Pfeiffer They would be mature

Bloom Yes

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[24]Norman, G F and Mittler A Interrelationship of Vitamin D and Sex Hormones in Calcium and Phosphorus Metabolism in Rats *Proc Soc Exp Biol and Med* 67 104 111 (1948)

[25]Urist M R Budy A M and McLean F C Unpublished experiments

Pfeiffer Then it might be a matter of ages I was wondering whether there wasn't some mechanism associated with the difference in response of the two species

Bloom On the other hand, the potentiality for bone growth is certainly there, Dr Pfeiffer It can be elicited by a fracture of one of the mouse bones Moreover, bone seeking radioactive isotopes go down into the bone and call forth in mice the same endosteal reaction as estrogen does but the metaphysis also becomes longer

Pfeiffer Do you have any explanation of the difference in response between mice and rats in relationship to estrogen?

McLean You have to go farther than mice and rats

Pfeiffer Oh yes I agree with you

McLean Outside of the birds, the only animals that we have found that respond specifically to estrogens are mice and rats We have surveyed a number of other species without being able to show any specific response

Pfeiffer Have you tried Xenopus the African clawed toad? It is possible that they may respond very much the same as the birds

Armstrong What about the human? I am sure Dr Albright would want to ask you about the human

McLean We have made no observations in man

Urist From the standpoint of Dr Albright's and Dr Reifenstein's metabolic studies, I think their conclusion was that the human reacted to estrogen like the mouse Is that correct?

Albright The woman is the same as the mouse qualitatively, if not quantitatively

Neuman Some day I am going to look this up but perhaps you people can save me time Is there a series of studies on the handling of calcium by the kidney? Is there a thorough clearance study to see if there is any special renal mechanism for calcium and the effect of hormones on this mechanism?

Howard I do not know, if you are looking at me

Copp We have done a little work on this subject, and found that the level of blood phosphate appears to have an effect on kidney clearance of calcium I shall be discussing this later

Neuman It seems to me that it is a terrifically important problem It has been neglected all these years It would be worth tackling

Armstrong Well, gentlemen, have you finished? If so we will adjourn for luncheon

OSTEOGENESIS[26]

FRANKLIN C McLEAN

From the Department of Physiology Division of Biological Sciences the University of Chicago Chicago Ill

Armstrong Dr McLean are you ready to open the discussion of the topic Osteogenesis ?

McLean Yes sir

I propose to keep my introductory remarks brief in order to allow time for Marshall Urist to present hitherto unreported work in some detail Some previous work either not mentioned or treated very briefly in my introduction will doubtless require attention in the discussion which will follow Dr Urist s presentation

The questions to be discussed in this session are old To some degree they were implicit in the observations of Duhamel on the healing of fractures in 1739-43[27] They became explicit in the experiments of Ollier on the transplantation of the elements of bone in 1858 68[28] Later contributions have added much to the subject both by improvement of techniques for observation and experiment and by clearer statements of the issues involved

The Concepts Involved in Osteogenesis

First a few words as to the issues It is clear that bone always arises as a result of the activity of cells which have differentiated from one variety or another of connective tissue cells When a cell has the ability to form bone it is said to have *osteogenetic potency* If this potency is brought into play *osteogenetic* activity is recognizable and if this activity is elicited by influences from outside the cell it is said to be *inducted* and the process is that of *induction* The substance—usually if not always hypothetical—which leads to induction is known as an *inductor* The property of *osteo*

This paper is based on work aided by grants from the Division of Research Grants and Fellowships of the National Institutes of Health U S Public Health Service from the Josiah Macy Jr Foundation and from the Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

[27] Duhamel H L Sur le Développement et la Crue des Os des Animaux *Mém Acad R des S* 55 354 (1742)

[28] Ollier L *Trait Expérimental et Clinique de la Régénération des Os* Paris Masson (1867)

genetic potency is shared by many if not all of the connective tissue cells of vertebrates the conditions which lead to exhibition of this potency have been the subject of many investigations and will receive the greater part of our attention today

It is not difficult by histologic observation to see the cells responsible for the formation of bone These cells have a characteristic morphology and are easily recognized as osteoblasts But it is not always easy to determine the origin of these cells and at what point and under what influence they are transformed into bone producing cells

Much of the histologic work on the problem of osteogenesis has been done by transplanting bone or elements of bone to soft tissues Here it is not always easy if living tissue is transplanted to determine whether further growth is from the transplant or from the tissues of the host or recipient Of course if dead tissue is transplanted and growth of bone follows it must arise from the cells of the host although the end product of the growth may be determined by the influence of the devitalized tissue

Osteogenetic activity may arise in embryonic life and may be transferred to the linear descendants of the cells originally displaying it Or this activity may be elicited from previously undifferentiated connective tissue cells by external influences—inductors—in response to a particular stimulus Thus we have in the case of the cell some of the same problems with respect to heredity and environment that we have for the organism as a whole

It has been pointed out that bone is a tissue but that bones are organs [29] The term potency may refer to the ability to form tissues in which case it is histogenetic potency or to the ability to form organs or organogenetic potency We shall hear more of this from Dr Urist

Resolution of the problems of osteogenesis is still further complicated by the fact that cells do not always do what they are capable of doing—a potency may be latent and exhibited only under certain special conditions

The Demonstration and Study of Osteogenetic Potency

We shall not dwell today on either the healing of fractures or the production of endosteal bone under the influence of estrogens Both of these subjects have been treated at length in former meetings of this Con

[29] Weinmann J P and Sicher H *Bone and Bones Fundamentals of Bone Biology* The C V Mosby Co St Louis (1947)

ference and of its predecessors It is desirable however to mention briefly some work that bears directly upon the subject matter of today s discussion

Heinen[30, 31] made tissue cultures from the bones of very young rats The outgrowth from the cultures was transplanted to the eyes of rats whereupon there was formed, in the eye not only osseous tissue but complete ossicles, adapted to the size and shape of the anterior chamber and filled with bone marrow This is a clear cut illustration of the transfer in tissue culture of osteogenetic potency from one generation of cells to their descendants Fell[32] using material from chicks demonstrated osteogenesis in tissue culture without the necessity of transplanting the cells from the culture to a more favorable environment Bloom[33] has demonstrated the ability of the bone marrow from pigeons to form bone in the anterior chamber of the eye when the host was under the influence of estrogens All of these examples bear witness to the ability of certain cells under certain conditions to exhibit osteogenetic potency and they illustrate also some of the methods that have been used in the demonstration and study of this property

Follis What was the original graft in Dr Heinen s studies Dr McLean?

McLean The implant was an outgrowth of a tissue culture of fetal bone or bone from newborn rats These were ordinary tissue cultures permitted to grow in a hanging drop Out from the culture connective tissue cells grow into the medium The central part of the culture was then removed and the outgrowth implanted into the anterior chamber of the eye

Hastings Which is the outside of the eye? Which is the corneal side?

Bloom The corneal side is out

Attempts to Isolate the Inductor Substance

McLean Another approach to the problem has been made use of in

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[30] Heinen J H Jr The *Differentiation of Bone and Cartilage from Tissue Cultures of Osteogenic Cells* Thesis (Ph D) University of Chicago

[31] Heinen J H Jr Unpublished work see illustrations in Maximow A A and Bloom W *Textbook of Histology* 5th Ed W B Saunders and Co, Philadelphia (1948)

[32] Fell H B Osteogenic Capacity *In Vitro* of Periosteum and Endosteum Isolated from the Limb Skeleton of Fowl Embryos and Young Chicks *J Anat* 66 157 (1932)

[33] Bloom William Unpublished work

recent years Levander[34] Annersten[35] Bertelsen[36] Lacroix[37] and others have shown that alcoholic extracts of bone when injected into the muscles of rabbits may give rise to ectopic bone formation All of these authors have postulated in one form or another a specific inductor substance for which Lacroix has proposed the name osteogenin It will be seen from Dr Urist s presentation that there is much more support from histologic studies for the concept of induction of bone formation in that it appears that under certain circumstances new bone actually arises from the cells of the host rather than from transplants of living cells which retain and exhibit their osteogenetic potency Unfortunately Heinen[38] has found that injection of alcohol alone in many instances is sufficient to induce bone formation in the muscles of rabbits and has reviewed the various circumstances under which rabbits form ectopic bone Heinen s work thus raises a question as to whether the results of the other authors cited constitute proof of the isolation of a specific inductor substance and final clarification of this subject will require further investigation

In introducing Dr Marshall Urist who will report on work done in the laboratories of Dr Bloom and myself I may say that this work began as an attempt to demonstrate the presence or absence of osteogenetic potency or osteogenetic activity in the cells of various tissues In the course of the work it became necessary to consider the role of induction in ectopic bone formation and to attempt not always successfully to distinguish between inherent osteogenetic potency and bone formation by induction in specific instances Dr Urist will present results of his work

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[34] Levander G A Study of Bone Regeneration *Surg Gynec & Obst* 67 705 (1938)

[35] Annersten S Experimentelle Untersuchungen uber die Osteogenese und die Biochemie des Fracturcallus *Acta chir scandinav* 84 1 (1940)

[36] Bertelsen, A Experimental Investigations into Postfœtal Osteogenesis *Acta orthop scandina* 15 139 (1944)

[37] Lacroix P *L Organisation des Os* Editions Liege (1949)

[38] Heinen J H Jr Dabbs G H and Mason H A The Experimental Production of Ectopic Cartilage and Bone in the Muscles of Rabbits *J Bone and Joint Surg* 31 765 (1949)

OSTEOGENETIC POTENCY AND OSTEOGENETIC INDUCTOR SUBSTANCES OF PERIOSTEUM, BONE MARROW, BONE GRAFTS, FRACTURE CALLUS, AND HYALINE CARTILAGE TRANSFERRED TO THE ANTERIOR CHAMBER OF THE EYE[39]

MARSHALL R URIST[40] and FRANKLIN C McLEAN[41]

From the Department of Physiology Division of Biological Sciences the University of Chicago Chicago Ill

Urist There are two reasons for showing this motion picture (1) a picture tells better than words how one can obtain a pure sample of fibro cartilaginous callus from a healing fracture (2) the fibrocartilaginous callus has great osteogenetic activity and is the best source of osteogenetic inductor substances

Description of Motion Picture Film

This motion picture shows how a sample of fibrocartilaginous callus, containing no bone can be isolated under a gross dissection microscope, under sterile conditions and transplanted to the anterior chamber of the eye We shall demonstrate that this tissue has (1) cells which have osteogenetic potency and (2) contains substances which induce new bone formation We shall postulate that the fibrocartilaginous callus (which consists of growing cartilage fibrocartilage and fibrous connective tissue cells) contains cells with the microscopic features of ordinary fibrous con nective tissue cells but possessing the property of osteogenetic potency in that they can differentiate into bone when transplanted into the anterior chamber of the eye

(Motion Picture)

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[39]These investigations were aided by grants from the Division of Research Grants and Fellowships of the National Institutes of Health U S Public Health Service from the Josiah Macy Jr Foundation and from the Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

[40]Consultant in Orthopedic Surgery Student Health Service University of California at Los Angeles Attending Specialist in Orthopedic Surgery Wadsworth General Medical and Surgical Hospital Veterans Administration Medical Center Sawtelle Los Angeles 24 California

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For the reasons mentioned by Dr McLean we used the rat in this experiment and avoided the rabbit a species in which bone formation can be provoked by the injury of a surgical operation In the rat however, it is not produced simply by *injuring the muscle tissue or by performing* a surgical operation on the animal s eye The operation can be done on the rat without sterile technique but asepsis is advisable because when it is used good implantation and successful results are more consistent

The tibia was fractured eleven days before this operation was done and the picture shows that we are amputating a leg that contains an eleven day healing fracture of the midshaft of the tibia The stump was closed with a *purse string suture The magnification of the pictures gets higher all the* time and with the increase of magnification the surgery appears to be more jerky

At eleven days of healing the fibrocartilaginous callus is a separate mass of tissue and has no contact with the growing bony parts of the callus The tissue was inserted with the use of a stylet into the angle of the anterior chamber between the cornea and the iris

Follis The same rat?

Urist The same rat All these are autogenous transplants

Histological sections were made of one half of the specimen that was put in the eye and the callus from which the specimen was cut in order to ascertain the original composition of the graft

Armstrong What would happen if you put these transplants in the peritoneal cavity?

Urist The same thing We did put them in muscle

Albright Would that allow a greater expansion of the mass? Does the mechanical limitation of space have any influence on the shape?

Urist Yes I think it determines the shape of the transplant but I think the size is determined by the donor tissue itself There is a slight increase in the size of the mass that is put in the eye but not very much

New bone can be cultured *in vitro* grown from transplants and has been described under pathological conditions in almost every organ of the body The nature of the principle which *initiates and sustains the process of new* bone formation is one of the most important problems of osteogenesis

Conclusions Re Osteogenetic Potency of Tissues

We have made observations upon the osteogenetic activity of 250 trans fers of different musculo skeletal tissue to the anterior chamber of the eye

The cellular changes and the end product of the transplants suggests a few conclusions and a great many questions

Osteogenetic potency, the capacity to produce new bone, is possessed by certain tissues of the body, such as bone marrow periosteum and cancellous bone, all of which contain cells which differentiate into bone promptly when transplanted

The periosteum and endosteum tissues are histogenetic, or productive of bone tissue Periosteum, in particular is a specialized structure not simply a limiting membrane It contains cells in the cambium layer when observed in a young growing animal, which have osteogenetic potency or cells which have already differentiated into osteoblasts

Bone marrow, on the other hand, is not only histogenetic and productive of bone tissue, but organogenetic The reticular cells of the bone marrow that is the connective tissue stroma of the bone marrow are capable of differentiating into a complete ossicle with a permanent cortex and a hematopoietic marrow Periosteum will produce bone tissue, but not a complete ossicle, whereas other tissues like bone marrow and the outgrowth of cancellous bone tissue, will produce a whole bone Cartilage and compact bone also produce new bone and a complete ossicle Compact bone grafts prepared by thoroughly scraping off all the marrow and periosteum have been studied in detail by P P H DeBruyn[42] of the University of Chicago Department of Anatomy

Conclusions Re Inductor Substances of Tissues

Cartilage and compact bone appear to initiate bone formation by means of a special mechanism Growing cartilage or bone matrix contains osteogenetic inductor substances which confer osteogenetic potency upon in growing perivascular connective tissue of the host The process can be viewed when the tissue is either injured or transplanted and destined to be absorbed

The term "inductor substance' is used in the embryological sense as proposed recently by Levander[43] of Sweden and Lacroix[44] of Belgium The term "induction , as these investigators define it, also called 'organizer phenomena', postulates that the earliest differentiation and later develop-

[42]DeBruyn P P H Bone Formation by Fresh and Frozen Transplants of Bone Bone Marrow and Periosteum, *Anat Rec* 99 188 (1947)

[43]Levander, G Tissue Induction *Nature* 155 148 (1945)

[44]Lacroix P *L Organization des Os* Masson et Cie, Paris (1949)

ment of tissues depend upon the interaction of chemical influences of one tissue upon a nearby tissue

One of the earliest appearances of this idea in the modern literature is found in the work of Barth in 1893 who described it with the old fashioned expression of death and resurrection Barth wrote that the transplant always dies and that the host connective tissues grow into the donor tissue and the new bone arises from something in the substance of the old bone

These of course were relatively gross observations and are difficult to interpret because the experiments were done on transplants of bone to *bone* *The histological* evidence of Barth s thesis can be seen better when bone and other skeletal tissues are transplanted to soft parts which have no possible contact with bone tissue

Evidence for Osteogenetic Potency and Inductor Substances

I would like to review and illustrate the evidence which we have found for the thesis that tissues from or near a growing bone contain cells which possess both osteogenetic potency and which produce in addition osteogenetic inductor substances It would be interesting to postulate further that inductor substances and the conditions for osteogenesis may be created during the absorption of almost any tissue in the body including soft tissues and that they may occur following a mechanical injury or disease in the same way as after transplantation

By the same technique which was illustrated in the motion picture we *have made autogenous transplants of all the different kinds of non osseous* motor skeletal tissues All of these tissues were recovered for histological study within one month—four to five weeks—following insertion in the anterior chamber of the eye (See Table I)

Normal muscle produced no bone within thirty days Injured muscle transplanted forty eight hours after the muscle was contused crushed with a hemostat produced no bone within thirty days Muscle plus fascia was also negative Fascia joint capsule tendo Achillis ear cartilage and the special types of cartilage of the semilunar cartilage of the knee joint were also transplanted The rat possesses *two centers of ossification in each one* of the semilunar cartilages of the knee joint We learned this only after old bone was discovered in the transplants It is not present in the young rat and develops only *after the animal is mature* and has the permanent skeleton There is one center in the anterior portion and one in the posterior portion of the semilunar cartilage Ossification of the semilunar cartilage also *occurs* under pathological conditions in the human

Although every one of these transplants was negative there is reason to believe that if we had followed these transplants for three or four or seven months that they too may have induced new bone formation The studies of Greene[13] of Yale and Dyer and Kelley[15] of the U S Public Health Service at Bethesda Maryland in which different kinds of embryonic and cancer cells were transplanted to the eye have shown that if a tissue is allowed to remain in the eye long enough it will produce bone formation

Periosteum bone marrow bone tissue epiphyseal cartilage articular cartilage fibrocartilaginous callus and devitalized fibrocartilaginous callus produce new bone consistently The process is shown in the photomicrographs (Fig 5 through 25) and tables (Tables I through VIII)

TABLE I

Autogenous Transfers of Miscellaneous Non Osseous Motor Skeletal Tissues

	Number of Transplants	Number Showing New Bone
Normal Muscle	10	0
Injured Muscle 48 Hours	2	0
Muscle and Fascia	2	0
Fascia	2	0
Tendon Achilles	4	0
Joint Capsule	2	0
Cartilage of the External Ear	10	0
Semilunar Cartilage Knee Joint	10	2 ( )

TABLE II

Periosteum

	Number of Transplants	Number Showing New Bone
Adult Rat	8	0
Immature Rat	8	4
24 Hours After a Fracture	5	1
48 Hours After a Fracture	8	2
72 Hours After a Fracture	8	4
96 Hours After a Fracture	2	2

TABLE III

Fracture Hematoma

	Number of Transplants	Number Showing New Bone
24 Hours	2	0
48 Hours	1	0
72 Hours	1	0
96 Hours	2	0
240 Hours	1	0

TABLE IV

Autogenous Transfers of Bone and Bone Marrow

Tissue	Number of Transplants	Number of Days in the Eye	Number Showing New Bone
Bone Marrow	5	28	2
Cancellous Bone	2	11	1
Cancellous Bone	2	25	1
Cancellous Bone	4	34	4
Cortical Bone	10	28-32	2
Cortical Fracture Ends	2	28	1

TABLE V

Autogenous Transfers of Fibrocartilaginous Callus from 10 to 12 Day Healing Fractures

Number of Successful Transfers	Number of Days in the Eye	Number Showing New Bone
1	6	0
3	7	0
3	8	0
2	9	0
5	10	3
5	11	4
1	13	1
3	17	2
1	18	1
1	23	1
1	27	1
1	30	1
7	35	7
2	36	2

TABLE VI

Autogenous Transfers of Devitalized Fibrocartilaginous Callus

Treatment	Number of Transplants	Number of Days in the Eye	Number Showing New Bone
Frozen	3	35	1
Boiled	2	35	2

TABLE VII

Autogenous and Homogenous Transfers of Epiphyseal Cartilage

Number of Transfers	Number of Days in Eye	Number Showing New Bone
Autogenous Transfers		
2	11	0
2	13	2
2	16	2
4	18	4
1	21	1
2	31	2
2	34	2
1	35	1
2	36	2
Homogenous Transfers		
7	27	7

TABLE VIII

Autogenous Transfers of Articular Cartilage

Number of Transplants	Days in the Eye	Number Showing New Bone
1	11	0
1	13	0
1	14	0
1	21	1
1	31	1
1	34	1
2	35	2
1	36	1



Fig 5 Photomicrograph Showing New Compact Bone Formation Five Weeks after Autogenous Transfer of Periosteum from the Tibia of Four Weeks Old Rat

(× 150) The absence of the Haversian systems may indicate that mechanical function of the bone tissue is necessary before the tissue can develop the characteristic structure of cortical bone



Fig 6 Photomicrograph Show ng a Fragment of tl e Caps ıle of the Knee Jo ı t ın the Angle of the Anterıor Chamber of the Eye

(× 65) The fragment s bet veen t e r s (ho vn belo v) and the cornea (sho vn abo e) Tl ere ıs no e dence *of new bone formatıon after 35 days* n the eye



Fig 7 Photo crograp h Sho v ıng a Fragme t of Muscle Trans ferred to the Anter or Chamber of tl e Eye

(× 5) There s n ev den e of new bone format on af er a fragn e t of muscle f om the belly of the ga t oc em u l as been the ante or chamber for 35 days No e tl e n flammato y prol fe a e con ect e t ssue react o of the host b cl e capsu es tl e mu cle t ssue a d con a ns masses of old fib n or hemos der n



Fig 8 Photomicrograph Showing a Fragment of the Achilles Tendon Transferred to the Anterior Chamber of the Eye

(× 65) After 35 days the tissue is enveloped in granulation tissue containing flakes of old fibrin and hemosiderin but there is no new bone formation



Fig 9 Photomicrograph Showing the End-Product of a Fragment of Bone Marrow in the Anterior Chamber of the Eye

(× 135) After 35 days a complete new spherical shaped ossicle has *formed* It contains active hematopoietic red bone marrow and a wall of compact bone



Fig 10 Photomicrograph Showing a Fragment of Spongy Bone from the Upper End of the Tibia in the Anterior Chamber of the Eye

nuclei or empty lacunae



Fig 11 Photomicrograph Showing a Full Thickness Fragment of Cortical Bone from the Shaft of the Tibia in the Anterior Chamber of the Eye

(× 250) *After 22 days there are empty lacunae in the old bone which stains poorly* in the upper part of the section The new bone which is metachromatic staining bone and fibrous in structure, is sharply demarcated in the lower portion of the picture



Fig. 12. Roentgenogram Showing a Fracture of the Midshaft of the Tibia



Fig. 13. Photomicrograph Showing the Entire Anterior Portion of the Callus of the Fracture Shown in Figure 12

(× 10) The histologic composition of the excised area is shown in Figure 14



Fig 14 Histologic Section of One Half of the Tissue Excised from the Fracture Callus Shown in Figures 12 and 13

This is a typical sample of fibrocart lag nous callus and conta s actively growing sp ndle shaped fibrous connective t ssue cells fibrocart lage and hyal ne cartilage



Fig 15 Photomicrograph Showing a Fragment of Fibrocartilaginous Callus Encapsulated in Granulation Tissue in the Anterior Chamber of the Eye

(× 135) After 7 days in the eye there is a sharp line of demarcation between the tissues of the donor and those of the host. The surface of the transplant shows metachromatic staining but there is as yet no ingrowth of new vessels or absorption of the transplant



Fig 16 Photomicrograph from Another Transplant of Fibrocartilaginous Callus under High Power Magnification Showing the Earliest Invasion of the Donor Tissue after 11 Days in the Anterior Chamber of the Eye

(× 270) The beginning new bone formation occurred with the first ingrowth of new blood vessels and in association with the absorption of the donor cells



Fig 17 Photomicrograph of a Transplant of Fibrocartilaginous Callus 17 Days after Transfer to the Anterior Chamber of the Eye

(× 120) At this time there is further ab orption of cartilage and new bone formation on the surface of the donor tissue



IS

21 Days after Transfer to the Anterior Chamber of the Eye

(× 165) Nearly all of the cartilage has been absorbed, and replaced by a spherical mass of new spongy bone



Fig 19 Photomicrograph of the End Product of a Transplant of Fibro cartilaginous *Callus 28 Days after Transfer to the Anterior Chamber of* the Eye

(× 120) The spongy bone shown in Figure 18 appears to have been reconstructed *into a shell of compact bone The new ossicle is filled with primitive bone marrow* cells and hematopoietic tissue

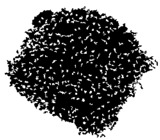


Fig 20 Roentgenogram of an Eye 35 Days after Transfer of 11-Day-Old Fibrocartilaginous Callus

Note the new ossicle formed in the angle of the anterior chamber The eye was adiologically positive for calcification only when it contained new bone formation



Fig 21 Photomicrograph Showing a Fragment of Fibrocartilaginous Callus which Had Been Devitalized by Boiling before Transfer to the Anterior Chamber of the Eye

(× 135) The fragment was devitalized by boiling at 100° C for 3 minutes before transfer After 36 days in the eye there is ingrowth of blood vessels and primitive connective tissue absorption of cartilage, and new bone formation just beginning within the surface of the donor tissue similar to the process in fresh viable transplants shown in Figure 17



Fig 22 Photomicrograph Showing a Fragment of Fibrocartilaginous Callus which Had Been Devitalized by Freezing before Transfer to the Anterior Chamber of the Eye

(× 135) The fragment was devitalized by freezing in liquid carbon dioxide before transfer After 36 days in the eye a scant deposit of new bone is formed on the surface of the transplant



Fig 23 Photomicrograph Showing a Fragment of Fresh Epiphyseal Cartilage from the Tibia of a 4 Weeks Old Rat after 18 Days in the Anterior Chamber of the Eye

(× 165) Vigorous new bone formation was produced similar to that obtained from fibrocartilaginous callus as shown in Figure 18





Fig 25 Photomicrograph Showing the Product of a Transfer of Articular Cartilage from the Upper End of the Tibia to the Anterior Chamber of the Eye of a Young Rat

(× 125) A new ossicle complete with an articular surface and active hematopoietic marrow has formed within 35 days in the eye



Fig 24 Photomicrograph Showing the Product of a Similar Transplant of Epiphyseal Cartilage after 36 Days in the Anterior Chamber of the Eye

(× 125) *The germinal cells of the cartilage persist on one side of the new ossicle which developed in a manner similar to that shown in Figures 9 and 19*

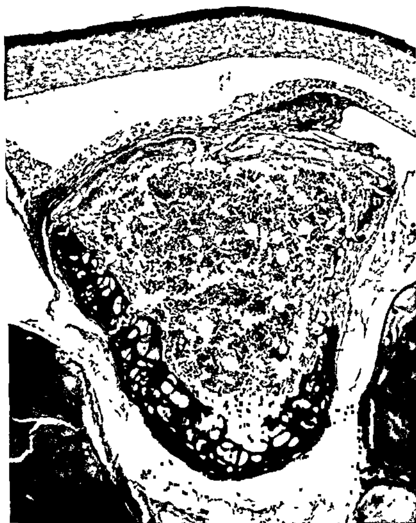


Fig 25 Photomicrograph Showing the Product of a Transfer of Articular Cartilage from the Upper End of the Tibia to the Anterior Chamber of the Eye of a Young Rat

(× 125) A new ossicle complete with an articular surface and active hematopoietic marrow has formed within 35 days in the eye

Conference Discussion

Sobel Has the perichondrium been stripped off?

Urist Yes, this is a thin slice obtained from the middle of the epi cartilages There is no calcification or bone in these specimens close to the row mother cells and found some of them in the graft enous transfers were just as active as autogenous transplants

The assumption in studies on transplantation—and there seem general agreement—is that in order for a cell to grow and prolife must have autonomy An homogenous tissue will survive transpla and it may live for a while but it does not proliferate On the othe a heterogenous transplant will not survive any time but simply und necrosis

Cancer cells were observed by Greene to proliferate, and this is the differences between the normal cells and the cancer cells

Follis Do those big cells have glycogen in them?

Urist I do not know We did not do glycogen stains

Hastings What do you mean by saying there is a joint surface?

Urist The picture shows a bone with an articular cartilage The mous cells on the top are the kind of cells that you have on the joint su

Hastings Are they new cells?

Urist I would not venture to say whether they are new cells or w they are the same ones we put in It is my opinion that these cells top are the ones we put in the eye and that the deeper cells conce might have developed since

What would you say about that Dr Bloom?

Bloom I would not know

Hastings I was just wondering what you meant by "joint"

Bloom What are those peculiar things to the left?

Urist That is cortical bone tissue but it contains remnants of articular cartilage cells

Some of these cells appear to be undergoing transformation o sorption

Follis What is that eroding the lens?

Urist What would you say that is, Dr Bloom?

Bloom I do not know

Urist It is a kind of debris Call it gurry That is the best description I can offer at the moment

Hastings What does that mean?

Urist It may be part of a degenerated lens

Pfeiffer You sectioned all of these?

Urist Yes it may be coagulated vitreous humor Perhaps degenerated lens In a good transplant the lens is intact You may have observed in the movie that the lens was over one side and the transplant to the other

Pfeiffer The lens usually remains unaltered in a good intraocular graft You have to damage the lens in making your transplant if the lens is to be harmed in any way If you do not damage it in making your transplant or get marked necrosis the lens remains just the same as it always was

What species did Dr Greene make his transplants in? I wonder if the experiments he was referring to were done in rats?

Urist Dr Greene's experiments have been done on rats guinea pigs rabbits and mice[46]

Pfeiffer I don t remember about rats specifically

Follis Is it the substance you put in or just the chronic inflammation?

Urist The tissue appears to supply the inductor although we believe that the tissue of the eye itself may produce the inductor following an injury such as an injection of formalin

Pfeiffer Don t you usually get necrosis before bone is formed under these circumstances?

Urist Yes strange as it may seem complete necrosis of a large area of tissue is not as good for making bone as are those tissues which show less damage

Pfeiffer If you get complete necrosis that is a different matter but partial necrosis is apparently tied up with it isn t it?

Urist Yes necrosis in an injured tissue may produce bone Osteogenesis seems to be a reaction of the body to either injury or transplantation of the tissue

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[46]Greene H S N The Use of the Mouse Eye in Transplantation Experiments *Cancer Research* 7 491 (1947) Identification of Malignant Tissues *J A M A* **137** 1364 (1948)

After freezing in liquid carbon dioxide fibrocartilaginous callus made bone After it was boiled, it made bone But if it was dried at room temperature in a sterile dessicator jar it did not make bone

Robinson How soon does it dry at room temperature?

Urist Forty eight hours The tissue was just a brown crumb when it was implanted in the eye

Robinson How long did you boil it?

Urist Three minutes

Robinson Did you dry it in a vacuum at low temperature?

Urist No The idea at the time was to devitalize the tissue by different physical methods If we had known what was going to happen we probably would have frozen it first and evaporated off the ice in the Altman Gersh apparatus in order to better preserve the enzyme content of the tissue

Follis You have the factor of autolysis there which you would not have with the other two Is that right?

Urist Yes and drying at room temperature is also very destructive to enzymes

Hastings Did you ever freeze anything except this cartilage?

Urist Yes we tested frozen epiphyseal cartilage

Hastings How about muscle?

Urist We did not test muscle or any other soft tissue

Shorr Dr Urist how prolonged was the boiling?

Urist Three minutes in 0 9 per cent saline solution

Johnson Didn t you say that if you get bone formation under thirty days you consider that to be real true specific osteogenesis?

Urist Yes if we found a formless mass of bone tissue no marrow no organization of the structure into a complete ossicle we assumed the donor tissue contained osteoblasts or cells with previously acquired osteogenetic potency such as those found in the cambium layer of the periosteum

Johnson Then when you use the formalin what kind of time span does that take?

Urist We did not fix any of our transplants with formalin

Johnson You said even formalin would produce bone in the eye

Urist Yes, it has been shown by Nobukatsu[47] that if a drop of formal dehyde is injected into the eye the eye itself will produce new bone

Johnson How long does that take?

Urist A month or longer, a longer period of time than the 10 days in which bone will be produced by young cartilage or bone

Bartter What is the shortest time found by Greene and Dyer for the development of bone in cancer transplants?

Urist I do not know the shortest time interval Greene stated[45] that he always found bone in his transplants if they were allowed to remain in the eye long enough Helen Dyer[45] suggested that three to seven months was the usual period of time

Bartter It does not occur before three months?

Urist Yes In Greene s letter he says very strongly that he almost always—I think he uses the word always —found bone in his transplants if he allowed them to remain for three to seven months No the way he said it was that if we allowed them to remain there long enough Helen Dyer says three to seven months

Bartter These maltreated specimens of yours produced their bone only after thirty days Is that right?

Urist Yes

Armstrong Does anyone wish to make any comments about this presen tation or to ask any questions? I am watching the clock and I know that we have other data which will bear specifically on bone formation that I should like to get before us this afternoon

Howard I should like to ask Dr Urist one thing In what pathological conditions do you get complications of the semilunar cortex with the osteo genetic center in it? I have never heard of that What is it?

Urist I shall be glad to send you an x ray of it

Howard I have seen it but what is the pathological condition?

Urist It occurs in rheumatoid arthritis quite frequently It has also been described as an isolated finding in the knee joint in one or both carti lages in patients who have had an injury but apparently no other disease[48]

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[47]Nobukatsu I Experimentelle Untersuchungen uber die Verknochung im Auge *Graefe s Ar l Ofthalmol* 125 267 279 (1930)

[48]Smille I S *Injuries of the Knee Joint* The Williams & Wilkins Co Baltimore Md (1946)

It is associated with periodic effusions of the joint and erosion of the articular surfaces

I have a case that I am treating now, a woman with rheumatoid arthritis who has ossification of both semilunar cartilages of her knee joint

Howard Is it ossification or calcification?

Urist I think it is ossification The reports in the literature on this subject state there are areas of new bone formation in the substance of the cartilage

Follis How common is it?

Urist It is not too uncommon in rheumatoid arthritis

Follis How many cases have you ever seen?

Urist I have seen five or six cases

Follis I have never seen it histologically in our surgical material

Urist I shall be glad to send you an x ray of a case showing it There are numerous articles on the subject in most of the journals on bone and joint surgery

Robinson How long was the bone frozen, at what temperature, and how rapidly?

Urist The sample of tissue was put under a blanket of carbon dioxide snow by the set up used with a freezing microtome It was kept under there for three minutes then allowed to thaw out in normal saline, and later put in the eye

Sobel May I ask a foolish question? You transplant cartilage Where do the osteoid cells come from after the transfer?

Urist That is the all important question The postulate is that growing cartilage and growing bone contain an inductor substance which will stimulate ingrowing perivascular connective tissue to differentiate into bone There is some question how all this happens It appears that new bone formation begins when the transplant is absorbed and that absorption of the tissue initiates osteogenesis After it is initiated it may be self sustaining All this is conjecture and requires further study

Hodge I should like to ask what the significance is of the location of the new bone It is always in the periphery, is it not?

Urist This is another important question

When the specimen of cartilage or bone is implanted in the eye it is first encapsulated in granulation tissue Then blood vessels with an adnexa of

connective tissue grow into it and absorb the donor cells At this time new bone formation begins on the surface of the donor tissue The capsule of the graft shows two layers an outer layer which is similar to the adventitious layer of the periosteum and an inner layer which is highly cellular It contains many blood vessels and actively proliferating connective tissue The bone is laid down first on the surface of the transplant As the bone formation proceeds the capsule becomes separated from the original donor cells Bone formation of course also occurs later inside of the absorption spaces of the donor tissue

The reaction goes to an end point which is the formation of an ossicle This is an organized process and is confined in a limited area by an envelope of connective tissue formed by the host

Sobel Does mineralization of the cartilage precede the formation of these osteoid cells or does it take place simultaneously?

Urist I do not know We tried to do some von Kossa stains on uncalcified material and the grafts would fall out of the block We have a few von Kossa stains but they are not very good I think we had just better leave that question to be answered by observations on good sections

I assume from looking at hematoxylin eosin and azure stains that the process is just like the endochondral calcification as it occurs everywhere in the skeleton

Shorr One thing you can say is that your inductor is heat stabile

Urist Yes the results suggest that boiled bone if it is not boiled too long will induce new bone formation Frozen bone and alcohol treated bone have been observed following transplantation into muscles of the rabbit by Nageotte and others

There is however a disturbing situation in this and many other such experiments in the literature in that the observations were limited to the rabbit in which it is easy to provoke bone formation simply by an injury

Pfeiffer At this point when we are talking about how easy it is to get bone formation in the rabbit it might be of interest to say that I had a student who repeated Annersten s[49] and Bertelsen s[50] experiments using the rat without getting bone formation from either bone extract that is alcohol extracts of bone or alcohol alone That has never been published

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[49] Annersten S Uber die Osteogenese be der Frakturheilung *Chirurg* **13** 76 (1941)

[50] Bertelsen A Experimental Investigations into Postfœtal Osteogenesis *Acta orthop scandina* **15** 139 (1944)

because the number of animals was not very great I was a little afraid to publish it without doing something more with it, but I am satisfied in my own mind that the rat is rather resistant to bone formation from these procedures, which makes this transplantation that you are doing in the eye all the more significant, it seems to me

Urist Dr Pfeiffer I would like to add that I do not find any evidence that the eye is any more receptive to bone formation than muscle tissue There may be tissues that may be more resistant to bone formation than the eye or muscle Dr Pfeiffer[31] has suggested the concept of host resistance to grafting in his recent paper on marrow transplants

McLean Dr Pfeiffer did you also do this on mice?

Pfeiffer Only on the testes of mice because I was limited by size of the animal I could not inject too much extract into the muscle of a rat without getting some of it to come in contact with the bone I was limited by size so I did not use much

McLean But you did inject the testes of the mouse

Pfeiffer Yes

McLean And you got no bone?

Pfeiffer No bone, that is in the early stages

I have over a hundred cases of mice in which I let them go for periods of seven and eight months I get something there which may be ossification or calcification, I am not sure which I have not studied the material I don t know I am not surprised that I get it It is the same thing that Harry Greene gets with these transplants in the eye that "stick around" for seven or eight months without growing, that is, autonomously You get bone formation, which is not surprising, and probably you would get bone formation here in these transplants if you let them go long enough, which as Dr Urist points out is unimportant, because that is something secondary to the problem we are studying

Asling The brain is highly receptive to transplants Of course, there, after an appropriate lapse of time, one can be certain that what one started out with as a bit of jelly from the embryo forms a pair of bones stimulating a radius and ulna and even a joint space can be identified

We are trying to standardize this so that we can perform grafts in hypophysectomized animals for the study of the hormones which control the morphogenesis of the limb bud

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31 Pfeiffer C A Development of Bone from Transplanted Marrow in Mice, *Anat Rec* 102 225 (1948)

Urist That is transplanting a whole limb bud

Asling That is taking a limb bud from an 11 day old fetus and putting it into a 21 day old (post natal) host

Urist There you are transplanting a limb bud both a tissue which has cells with osteogenetic potency and primitive cartilage which supplies the inductor These tissues have the capacity to go to the end point of the osteogenetic reaction

Sobel Do you know whether anyone has tried to stain for enzymes and other compounds that have been considered in bone formation such as phosphatase phosphorylase and possible glycogen and chondroitin sulfate to see whether all these are present in this type of calcification just as they appear to be present in normal calcification?

Urist I do not know of any studies that have been done in this direction This is certainly a fertile field for histochemistry I think that histochemical studies on this kind of material would be quite interesting and important

Levander[52] reported that the inductor is most concentrated in the non saponifiable fraction of his alcoholic extracts and is a fat soluble substance Is that right Dr McLean?

McLean I did not get the question

Urist Did Levander and Lacroix have the idea that this is a fat soluble substance?

McLean That is right

Urist May we assume that they have used water soluble extracts?

McLean Yes they used water soluble extracts with negative results Bertelsen did those too[50] My impression is also that when they extracted with alcohol and then got rid of the alcohol before injection their results were again negative

Copp Has this work ever been done on rachitic animals?

Armstrong You mean the transplants into the eye?

Copp Yes

I was wondering whether you could get development of osteoid matrix

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[52] Wallenstadt H Levander G and Hult L Studies on Osteogenesis *Acta orthop scand nav* 19 419 (1950)

Urist Yes Heinen did that

McLean Heinen implanted some tissue culture cells into the eyes of rachitic animals and got an osteoid tissue

Copp Was there any calcification in it?

McLean No He got osteoid tissue

Copp Very good

Follis Doesn t the fact that bone will develop in injured muscle make you feel that possibly this is all just irritation that is myositis ossificans as a result of trauma without any adventitious material being produced?

Urist Is myositis ossificans a degenerative disease of muscle?

Follis Let s not talk about that because I do not know what that is but let s talk about areas that one knows have been traumatized in which muscle must have been injured where one gets true bone and where there has been nothing introduced from without

Hastings It does not take longer than 30 days?

Follis I think it probably does

Howard Not necessarily Dr Follis I do not think Certainly one gets calcification there that quickly

Johnson One can get calcification within 10 or 12 days but the first calcification is not calcification of bone it is calcification of the damaged muscle The first bone forms in approximately 30 days

Urist Yes that is true When alcohol is injected into a rabbit s muscle some kind of an amorphous calcification occurs in surrounding tissue along with actual bone formation I don t know whether it precedes bone formation but it is certainly an associated phenomenon

Follis Don t you have material Dr Johnson from irritation of rifle straps and that sort of thing?

Johnson We have somewhere in the order of 150 cases of human material that range from a few hours after injury up to 30 years after injury and it is from this material that I made the statement The calcification appears about five days on section and about ten days on x ray, is actually laid down as cross striations of the da[illegible] d muscle so that calcium comes out as striations That all clea[illegible] oliferati[illegible] it with bone the earliest bone formation [illegible] out a [illegible]

Hastings That should not surprise an[illegible] d I heart in fib[illegible] ventricle that were

pletely anoxic It just calcified the fiber and the cross striations looked as if they were a marble lattice I think that is because when the organic phos phate breaks down the inorganic phosphate comes out meets the calcium of the extracellular fluid and calcium phosphate precipitates

Johnson In the human heart it is interesting that this seems to occur only in uremia where one does not have calcium as a factor since blood calcium is depressed but where blood phosphate is considerably elevated

A FURTHER CONSIDERATION OF THE EFFECTS OF BERYLLIUM SALTS ON *in Vitro* CALCIFICATION OF CARTILAGE[53]

ALEXANDER B GUTMAN[54] and T F YÜ

From the Columbia Research Service Goldwater Memorial Hospital and the Department of Medicine Columbia University College of Physicians and Surgeons New York City

Armstrong We are going along now to Dr Gutman s presentation which bears on some of the details of bone formation He will be making of course a continuation report of work carried out since the last meeting of this Conference

Gutman In previous Conferences of this group[55 56 57] I reviewed the evidence implicating the complex series of enzymatic reactions characterizing phosphorylative glycogenolysis as part of the preparatory phase of *in vitro* calcification of cartilage when phosphorus is provided as inorganic phosphate The glycogenolytic cycle could be traced to the point of phosphopyruvate formation since like glycolysis in liver muscle and other tissues *in vitro* calcification of cartilage is blocked by the enolase inhibitor 10^{-4} M flouride

The further fate of the phosphorus of phosphopyruvate the mechanism by which it presumably is made available for formation of the bone salt has not been elucidated It was pointed out[57] that this might occur by the immediate *dephosphorylation* of phosphopyruvate ostensibly resulting in a localized concentration of phosphate ion and precipitation of bone salt much in the manner suggested by Robison[58] Another possibility more

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[53]These studies were supported in part by a gift from the John A Hartford Foundation.

[54]Present address The Mount Sinai Hospital New York 29 N Y

[55]Gutman A B Relation of Phosphorylase and Phosphatase to Calcification in Cartilage *Trans Macy Conference on Metabolic Aspects of Convalescence* 14 20 (1946)

[56]Gutman A B and Yu T F Further Studies of the Relation Between Glycogenolysis and Calcification in Cartilage *Trans Macy Conference on Metabolic Interrelations* 1 11 (1949)

[57]Gutman A B and Yu T F A Concept of the Role of Enzymes in Endochondral Calcification *Trans Macy Conference on Metabolic Interrelations* 2 167 (1950)

[58]Robison, R The Possible Significance of Hexosephosphoric Esters in Ossification *Biochem J* 17 286 (1923)

favorably considered[57] was *transphosphorylation* i e, direct transfer of the phosphorus of high energy phosphopyruvate to some unidentified acceptor without passing through the stage of phosphate ion The phosphate acceptor in question might be the ultimate (as yet unknown) substance in the cartilage matrix undergoing calcification or more likely (by analogy with events in other tissues) an intermediary phosphoric ester or esters Possible intermediaries considered were adenosine polyphosphate and creatine phosphate[57]

The role of alkaline phosphatase in the processes of endochondral calcification also is obscure It would seem evident that this enzyme does not effect significant dephosphorylation of the phosphoric esters of the glycogenolytic cycle prior to phosphopyruvate formation The possibility was considered that alkaline phosphatase might act upon phosphopyruvate either as a dephosphorylase or in view of the phosphotransferase activity of some phosphatases[59, 60, 61, 62] as a transphosphorylase In muscle this transfer is catalyzed by adenosine diphosphate or adenylic acid transphosphorylases but the presence of these enzymes in calcifying cartilage has not been demonstrated

Effect of Specific Inhibitors of Alkaline Phosphatase

A new and promising point of attack upon this problem of the role of alkaline phosphatase in endochondral calcification would be to study the effects of a potent relatively specific inhibitor of alkaline phosphatase on *in vitro* calcification of cartilage Such an inhibitor was not available however until 1949 when Klemperer *et al*[63] and Grier *et al*[64] published their studies on the marked and apparently highly selective inhibition of intestinal bone and other tissue alkaline phosphatases by beryllium salts Acting upon a suggestion by Dr Joseph C Aub at our Conferences last year Dr Yu and I undertook this new approach to the problem The initial results of our work have appeared elsewhere[65] but I should like to

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[59] Axelrod B A New Mode of Enzymatic Phosphate Transfer *J Biol Chem* 172 1 (1948)

[60] Axelrod B A Study of the Mechanism of Phosphotransferase Activity by Use of Radioactive Phosphorus *J Biol Chem* 176 295 (1948)

[61] Meyerhof O and Green H Transphosphorylation by Alkaline Phosphatase in the Absence of Nucleotides *Science* 110 503 (1949)

[62] Meyerhof O and Green H Synthetic Action of Phosphatase II Transphosphorylation by Alkaline Phosphatase in the Absence of Nucleotides *J Biol Chem* 183 377 (1950)

[63] Klemperer F W Miller J M and Hill C J The Inhibition of Alkaline Phosphatase by Beryllium *J Biol Chem* 180 281 (1949)

[64] Grier R S Hood M B and Hoagland M B Observations on the Effects of Beryllium on Alkaline Phosphatase *J Biol Chem* 180 289 (1949)

[65] Yu T F and Gutman A B Effect of Beryllium on *In Vitro* Calcification of Cartilage *Proc Soc Exper Biol and Med* 75 481 (1950)

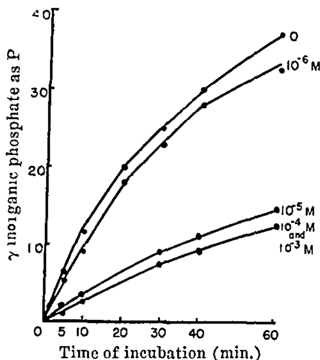


Fig 27 Time Activity Curves for Inhibition of Cartilage Homogenate Alkaline Phosphatase Activity by Beryllium in Various Concentrations

... final pH 9 6, the tempera
...d Gutman A B Effect
...oc *Exper Biol and Med*

cartilage The technique employed was the same as previously described except that additional measures were instituted to minimize loss of CO_2 and thus changes in pH during incubation a layer of mineral oil was added to the incubating fluid and 10 ml volumetric flasks (long necked) were employed for incubation

In experiments with *β glycerophosphate as the sole source of phosphorus* in amounts equivalent to 10 mg % P, calcification was completely inhibited by beryllium in concentrations of 1.0×10^{-3} M 1.0×10^{-4} M and 1.0×10^{-5} M Definite calcification, though somewhat less than observed in control solutions devoid of beryllium was obtained in the presence of 1.0×10^{-6} M Be (Figure 28) Good calcification, with no inhibition whatever was observed in the presence of 1.0×10^{-7} Be The critical concentration of beryllium necessary to block calcification in these experiments was thus found to be between 1.0×10^{-5} M Be and 1.0×10^{-6} M Be There is therefore a striking correspondence between the minimal concentration of beryllium salts required to block *in vitro* calcification of cartilage when phosphorus is supplied solely as β glycerophosphate (10 mg % P) and the minimal concentration necessary to effect marked inhibition (64%) of cartilage homogenate alkaline phosphatase Results similar to those with β glycerophosphate were obtained when sodium phenylphosphate (10 mg % P) or creatine phosphate (20 mg % P) was employed as the sole source of phosphorus in the incubating solution

When *inorganic phosphate was employed as the sole source of phosphorus* (6 mg %) quite different results were obtained (Figure 28) No inhibition of *in vitro* calcification was observed in the presence of 1.0×10^{-6} M Be, 1.0×10^{-5} M Be or 1.0×10^{-4} M Be, although 1.0×10^{-3} M Be

words, in the presence of enough

72% of the alkaline phosphatase

lock calcification with β glycero

phosphate (10 mg % P) entirely, calcification with inorganic phosphate apparently was completely unaffected The inhibition of calcification by 1.0×10^{-3} M Be probably is not related to effects on phosphatase since in this higher concentration beryllium presumably is not as selective in its action on enzymes and, as already indicated there is no significant difference in the inhibition of cartilage homogenate phosphatase within the range 1.0×10^{-4} M Be and 1.0×10^{-3} M Be, it is assumed either that some inorganic phosphate is removed as insoluble beryllium phosphate or that some essential enzyme which is neither alkaline phosphatase nor one of those in the glycolytic system (*vide supra*) is blocked

Of further interest was the finding that *in the presence of both β* glycerophosphate (10 mg % P) and *inorganic phosphate* (6 mg % P) the results obtained were indistinguishable from those with inorganic phosphate alone

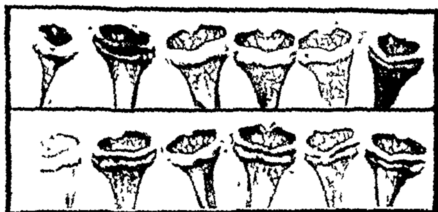


Fig 28 The Effect of the Source of Phosphorus on the Inhibition of Cartilage Homogenate Alkaline Phosphatase Activity by Beryllium in Various Concentrations

Top row from left to right 1 Control slice incubated in basal salt solution (B S S) *without* calcium or phosphorus note wide zone of uncalcified cartilage 2 Control slice incubated in B S S *plus* calcium *plus* β glycerophosphate (10 mg % P) note good calcification 3 4 5 6 Slices incubated in B S S *plus* calcium *plus* β glycerophosphate (10 mg % P) *plus* beryllium 10^{-3} 10^{-4} 10^{-5} and 10^{-6} M respectively note complete inhibition of calcification in slices 3 4 and 5 but definite calcification with only partial inhibition in slice 6

Bottom row from left to right 1 Control slice incubated in B S S *without* calcium or phosphorus note wide zone of uncalcified cartilage 2 Control slice incubated in B S S *plus* calcium *plus* inorganic phosphate (6 mg % P) note good calcification 3 4 5 6 Slices incubated in B S S *plus* calcium *plus* inorganic phosphate (6 mg % P) *plus* beryllium 10^{-3} 10^{-4} 10^{-5} and 10^{-6} M respectively note complete inhibition of calcification in slice 3 but good calcification in slices 4 5 and 6

We did not encounter the interference phenomenon previously described[37] with ATP and ADP

The implication of these experiments is that alkaline phosphatase is essential for *in vitro* calcification of cartilage when phosphorus is supplied in the form of certain *phosphoric esters* (β glycerophosphate phenylphosphate creatine phosphate) the phosphorus of these esters presumably being made available for calcification by phosphatase in the manner suggested by Robison On the other hand alkaline phosphatase activity appears not to be essential for *in vitro* calcification of cartilage at least as a dephosphorylating enzyme when phosphorus is supplied as *inorganic phosphate* the condition presumably obtaining in endochondral calcification *in vivo*

Additional Experiments Affecting Interpretation of Effect of Beryllium

This interesting conclusion however is subject to reservation because of the results of additional experiments some of which may be contradictory although most are I think understandable within the framework of our interpretation For example in the *in vitro* calcification experiments with β glycerophosphate if the concentration of ester is increased to 20 mg % P the inhibitory effect of beryllium salts is reduced no impairment of calcification whatever is noted in concentrations of $1\,0 \times 10^{-6}$ M Be or $1\,0 \times 10^{-5}$ M Be definite inhibition but some calcification with $1\,0 \times 10^{-4}$ Be and complete blocking of calcification only with $1\,0 \times 10^{-3}$ M Be This result is consistent with the observations of Klemperer[43] who found decreasing inhibition of alkaline phosphatase activity by beryllium when increasing concentrations of phenylphosphate substrate were used

More troublesome are experiments in which glucose 1 phosphate glucose 6 phosphate or fructose diphosphate was used as the sole source of phosphorus in *in vitro* calcification experiments (Figure 29) With *glucose 1 phosphate as the sole source of phosphorus* in concentrations of 10 mg % P calcification was completely inhibited by beryllium in concentrations of $1\,0 \times 10^{-6}$ M $1\,0 \times 10^{-5}$ M $1\,0 \times 10^{-4}$ M and $1\,0 \times 10^{-3}$ M When the amount of glucose 1 phosphate was doubled to 20 mg % P there was some inhibition but more or less distinct calcification in the presence of $1\,0 \times 10^{-6}$ M and $1\,0 \times 10^{-5}$ M Be calcification was completely blocked by $1\,0 \times 10^{-4}$ M and $1\,0 \times 10^{-3}$ M Be With *glucose 6 phosphate as the sole source of phosphorus* in concentrations of 20 mg % P (it is difficult to obtain satisfactory calcification in control slices with 10 mg % P) more or less distinct calcification was obtained in the presence of $1\,0 \times 10^{-6}$ M and $1\,0 \times 10^{-5}$ Be calcification was completely blocked by $1\,0 \times 10^{-4}$ M and $1\,0 \times 10^{-3}$ M Be With *fructose disphosphate as the sole source of phosphorus* in concentrations of 10 mg % P slight but definite calcification

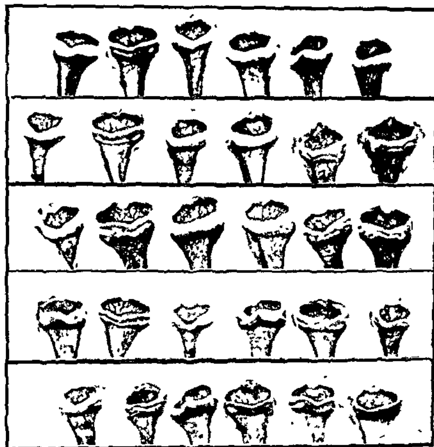


Fig 29 The Effect of the Source of Phosphorus on the Inhibition of Cartilage Homogenate Alkaline Phosphatase Activity by Beryllium in Various Concentrations

Row 1 (top) from left to right 1 Control slice incubated in basal salt solution (B S S) *without* calcium or phosphorus note wide zone of uncalcified cartilage *2* Control slice incubated in B S S *plus* calcium *plus* glucose 1 phosphate (10 mg % P) note good calcification *3 4 5 6* Slices incubated in B S S *plus* calcium *plus* glucose 1 phosphate (10 mg % P) *plus* beryllium 10^{-3}, 10^{-4} 10^{-5}, and 10^{-6} M respectively note complete inhibition of calcification

Row 2 from left to right 1 Control slice incubated in B S S only *2* Control slice incubated in B S S *plus* calcium *plus* glucose 1 phosphate (20 mg % P) note good

5 and 6.

was observed in the presence of 1.0×10^{-6} M and 1.0×10^{-5} M Be, calcification was completely blocked by 1.0×10^{-4} M and 1.0×10^{-3} M Be. When the amount of fructose disphosphate was doubled to 20 mg % P, marked calcification took place in the presence of 1.0×10^{-6} M, 1.0×10^{-5} M and 1.0×10^{-4} M Be, block occurring only with 1.0×10^{-3} M Be; these experiments (the only ones in this series) closely resembled the results when phosphorus was supplied only as inorganic phosphate.

It was anticipated that the effect of beryllium in experiments utilizing phosphoric esters in the glycogenolytic series would be substantially the same as when phosphorus is supplied in the form of inorganic phosphate, since utilization of inorganic phosphate for *in vitro* calcification of cartilage would appear to involve synthesis of these esters. This clearly was not our finding, however, and indeed the results are so variable and complicated that we are unable to interpret them beyond stating that they are not as predicted. Whether this means that our initial interpretation of the beryllium experiments was incorrect, or that secondary reactions of these phosphoric esters affect the course of the experiments is not clear.

Summary of Interpretation

The overall effects of beryllium salts on *in vitro* calcification do make it even more evident, however, that the enzyme mechanisms involved in the utilization of inorganic phosphate for endochondral calcification differ significantly from those operating in the utilization of β glycerophosphate and other phosphoric esters which are not in the glycogenolytic series but are dephosphorylated by alkaline phosphatase. As already indicated,[56, 67] 10^{-3} M phlorizin, 10^{-3} M iodoacetate and 10^{-4} M fluoride, all inhibitors of the glycogenolytic cycle, block *in vitro* calcification with inorganic phosphate but do not affect calcification with β glycerophosphate or significantly inhibit alkaline phosphatase (Tables IX and X), whereas beryllium in concentra

[67] Gutman, A. P., Warrick, F. B., and Gutman, E. B.: Phosphorylative Glycogenolysis and Calcification in Cartilage, *Science* 95: 461 (1942).

note complete inhibition of calcification in slices *3* and *4* but slight calcification in slices *5* and *6*.

Row 5 (bottom), from left to right: *1*, Control slice incubated in B.S.S. only; *2*, Control slice incubated in B.S.S. *plus* calcium *plus* fructose 1,6 diphosphate (20 mg % P); note good calcification. *3, 4, 5, 6*, Slices incubated in B.S.S. *plus* calcium *plus* fructose 1,6-diphosphate (20 mg % P) *plus* beryllium 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M respectively; note complete inhibition of calcification in slice *3* but good calcification in slices *4*, *5* and *6*.

TABLE IX

Mean Percent Inhibition of Alkaline Phosphatase Activity of Rat Cartilage Homogenates by Beryllium Salts, Phlorizin Iodoacetate, Fluoride and Cyanide

(*Substrate 1% β glycerophosphate, buffer veronal, final pH 9.6,* time 0.5 hr, temp 37° All control cartilage preparations standardized per 0.2 ml sample to split off 10.20 γ P as inorganic phosphate under these conditions)

	Inhibitor Added	Concentration (Moles)	% Inhibition of Alkaline Phosphatase Activity
1	Beryllium (sulfate)	1×10^{-7}	0
		1×10^{-6}*	12
		1×10^{-5}*	64
		1×10^{-4}*	72
		1×10^{-3}*	72
2	Phlorizin	5×10^{-3}	0
		1×10^{-2}*	9
		2.5×10^{-2}	28
		5×10^{-2}	46
3	Iodoacetate	2×10^{-4}	0
		1×10^{-3}*	0
		5×10^{-3}	0
4	Fluoride	2×10^{-5}	11
		1×10^{-4}*	9
		5×10^{-4}	6
5	Cyanide	2×10^{-4}	10
		1×10^{-3}*	49
		5×10^{-3}	82

*Commonly employed concentrations

Effect of Phlorizin Iodoacetate, Fluoride and Beryllium on *In Vitro* Calcification of Cartilage when Phosphorus is Supplied as Inorganic Phosphate and as Various Phosphoric Esters

Source of P	Phlorizin 10^{-2} M	Iodoacetate 10^{-3} M	Fluoride 10^{-4} M	Beryllium 10^{-3} M	Beryllium 10^{-4} M	Beryllium 10^{-5} M	Beryllium 10^{-6} M
I Inorganic Phosphate (6 mg %)	0	0	±	0	+++	+++	+++
II Phosphoric esters in the glycogenolytic series							
a Glucose 1 phosphate (10 mg %)	+++	±	±	0	0	0	0
Glucose 1 phosphate (20 mg %)	Not done	Not done	Not done	0	0	+	++
b Glucose 6-phosphate (20 mg %)	+++	±	±	0	0	+	++
c Fructose 1 6-diphosphate (10 mg %)	+++	+	±	0	0	+	+
Fructose 1 6-diphosphate (20 mg %)	Not done	Not done	Not done	0	++	+++	+++
d 3 phosphoglycerate (20 mg %)	Not done	++	0		Not done		
III Phosphoric esters not in the glycogenolytic series but dephosphorylated by alkaline phosphatase							
a β glycerophosphate (10 mg %)	+++	+++	+++	0	0	0	++
β-glycerophosphate (20 mg %)	Not done	Not done	Not done	0	++	+++	+++
b Phenylphosphate (10 mg %)	+++	+++	+++	0	0	0	+++
c Creatinphosphate (20 mg %)	±	+++	++	0	0	+	+++

tions of 1.0×10^{-4} M and 1.0×10^{-5} M markedly inhibits alkaline phosphatase and blocks calcification with β glycerophosphate (10 mg % P) but does not affect calcification with inorganic phosphate (Tables IX and X). The results with phosphoric esters in the glycogenolytic series are not so clear cut (Table X). The effects of 10^{-2} M phlorizin, 10^{-3} M iodoacetate and 10^{-4} M fluoride are very much as anticipated from the relative position of these esters in the glycogenolytic cycle but the effects of beryllium are erratic and difficult of interpretation.

Conference Discussion

Follis You stated that time activity curves indicated very rapid inhibition of alkaline phosphatase of rat cartilage homogenates by beryllium within five minutes. Does that differ from the rate of inactivation of enzymes in general by inhibitors?

Gutman I am not prepared to say.

Hastings There are some which take quite a little time.

Follis A matter of a half hour.

Hastings Certainly 15 minutes. You get an S shaped curve.

Bezelinder Did I understand that addition of magnesium would overcome the inhibiting effect of beryllium on alkaline phosphatase, and also the addition of more β glycerophosphate?

Gutman Yes, the inhibiting effect of beryllium may be overcome at least in part either by adding more magnesium or by increasing the concentration of substrate. The effect of magnesium is understandable in terms of competition of ions, beryllium competing with the magnesium or manganese of the enzyme. The effect of adding substrate I believe is a phenomenon encountered with many enzyme systems.

Hastings Under your conditions of maximal inhibition by beryllium there was still some 25% of the alkaline phosphatase activity left?

Gutman That is correct.

Hastings Is it possible that this residual phosphatase activity was sufficient for calcification with inorganic phosphate?

Gutman I have no way of excluding that possibility. However, this same residue of alkaline phosphatase activity was insufficient to permit calcification when phosphorus was supplied as β glycerophosphate, phenyl phosphate or creatine phosphate.

Neuman Did you demonstrate the inhibition of the phosphatase in the slice? There are considerable amounts of old preformed calcification present which has a terrific affinity for beryllium I do not see how you can be sure that the concentration of beryllium in these solutions in the presence of this mineral would remain 10^{-5} and 10^{-4} M

Hastings You did not soak the slices in beryllium solution first without calcium and phosphorus and then see what would happen in the calcifying medium containing calcium and phosphorus?

Gutman In our earlier experiments we did not Later we pretreated cartilage slices for 15 minutes at 37° C with 10^{-6} 10^{-5} 10^{-4} and 10^{-3} M Be then washed the slices and incubated with calcium and β glycerophosphate (10 mg % P) Inhibition of calcification was observed and in about the same degree as when beryllium was added directly to the incubating medium When the same pretreatment with beryllium salts preceded incubation with inorganic phosphate calcification occurred in all slices except those exposed to 1.0×10^{-3} M Be just as if beryllium were present in the regular incubating medium

Hastings What were the relative concentrations of phosphorus in the experiments using phosphoric esters and inorganic phosphate?

Gutman Inorganic phosphate was regularly employed as phosphate buffer solution pH 7.4 containing 6 mg % P To get equivalent degrees of calcification with organic phosphoric esters it is necessary to have more phosphorus as phosphoric ester present 10 mg % P or more depending on the ester used In our experiments therefore there was always more phosphorus present as organic phosphoric ester than as inorganic phosphate Nevertheless the inhibiting effect of beryllium was always more pronounced in the ester experiments than in those with inorganic phosphate

Shorr Would you state again why you think that blocking of calcification with inorganic phosphate by 10^{-3} M Be does not indicate a specific inhibition of alkaline phosphatase?

Gutman First because there was no significant difference between the degree of inhibition of cartilage homogenate alkaline phosphatase between the range 1.0×10^{-4} M Be and 1.0×10^{-3} M Be whereas the difference in effects on *in vitro* calcification of cartilage slices by beryllium concentrations over this range was very striking when inorganic phosphate was used Secondly 10^{-3} M is an appreciable concentration of beryllium and although I know of no enzyme other than alkaline phosphatase markedly inhibited by 1.0×10^{-3} M Be few enzyme systems have been tested as yet in this regard Thirdly in this concentration of beryllium some of the inorganic phosphate may be removed from solution in the form of the very insoluble beryllium phosphate

Hastings You were working with about two millimoles per liter of phosphate?

Gutman Yes

Hastings And beryllium is about two millimoles to the liter So you were getting into the region where you were using up phosphate so it was no longer working with your ester

Neuman In the case of the ester, the same argument is effective We have been able to demonstrate a good complexing relationship between beryllium and ester phosphate At the 20 mg per cent phosphorus level beryllium would be largely in the form of a complex I doubt whether it would be effective in inhibiting enzymes In fact one does not need phosphorus around to inhibit the action of beryllium The solubility of beryllium in pure water is something in the order of 10^{-7} moles/liter because of the hydrolysis of Be^{++} to the hydroxide Therefore the only way beryllium can be introduced would be in the form of a complex anyway

McLean Did you have bicarbonate in your incubating solution?

Gutman Yes our solutions contain 220 meq sodium bicarbonate per liter

INHIBITION OF ENDOCHONDRAL CALCIFICATION *in Vitro* BY BERYLLIUM AND L HISTIDINE[68]

HOWARD H HIATT[69] and EPHRAIM SHORR

From the Department of Medicine Cornell University Medical College the Russell Sage Institute of Pathology and the New York Hospital New York N Y

Armstrong I think that it would have been nice when I had planned these programs if I had known exactly what everyone was going to talk about I then could have arranged things in a more systematic manner For example this would be a very good time for Dr Neuman to tell about his work However he is scheduled for tomorrow s session and I am sure that he will have a great deal to offer which will bear on this point

I am going to ask Dr Hiatt if he will give his paper now On account of the lateness of the hour his presentation will be the last of the afternoon

Hiatt We have been interested in the enzymatic aspects of *in vitro* calcification of rachitic rat epiphyseal cartilage Results of some of our preliminary experiments involving inhibition of this process by beryllium and L-histidine are intimately related to the material just presented by Dr Gutman These results will be reported chiefly to emphasize two critical questions

1) Are the results obtained in a system inhibited by beryllium or L histidine compatible with the concept that phosphorus is de posited in the bone matrix only after it has traversed the phos phorylative glycolytic cycle?

2) Is the interference with calcification observed with beryllium and L histidine the result of their inh bition of alkaline phosphatase?

Methods

Our method for studying *in vitro* calcification was described in detail in these proceedings[70] one year ago The incubating solution contained 5 meq KCl 70 meq NaCl and 22 meq $NaHCO_3$ calcium 9 6 mg % (as calcium

[68]This investigation was supported in part by a research grant from the National Institutes of Health U S Public Health Service

[69]U S Public Health Service Post Doctorate Research Fellow

[70]Marks P A and Shorr E Factors Wh ch Regulate the Depos tion of Calc um and Strontium in Rachitic Cartilage *In Vitro Trans Macy Conference on Metabolic Interrelations* 2 191 (1950)

chloride) and phosphoric ester, the latter in an amount calculated to yield 10 mg % phosphorus after complete hydrolysis When inorganic phosphorus was used it was added as a Na_2HPO_4 NaH_2PO_4 buffer pH 7 4 in amounts of 5 mg % L-histidine was used as the hydrochloride salt neutralized with NaOH before addition to the incubating solution Inorganic phosphorus determinations were performed according to the method of Fiske and Subbarow [71]

Bodansky has shown that whereas glycine and other *a* amino acids in very low concentrations increase the activity of alkaline phosphatases [72] *high concentrations exert an inhibitory effect* [73] *He noted that this inhibition* which for bone phosphatase is non competitive in nature depends upon intact amino and carboxyl groups in the amino acid L histidine[74] was found to exert a particularly great inhibitory effect on rat bone phosphatase with 0 002 to 0 003 M *L histidine causing a 50% inhibition of phosphatase* activity This inhibition was felt to result from the binding by the amino acid of an essential metal component of the enzyme a component which mediates the activation of the enzyme by magnesium [75]

Inhibition of alkaline phosphatase by beryllium has been demonstrated recently by Klemperer et al [76] by Grier et al [77] and by Dr Gutman [78] the latter extending these observations to rat epiphyseal cartilage phosphatase Replacement of magnesium by beryllium is thought to be the mechanism of action of this inhibitor [76 77]

Results with Beryllium

Our results with beryllium have been similar to those just described by

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[71]Fiske C H and Subbarow Y The Colorimetric Determination of Phosphorus *J Biol Chem* 66 375 (1925)

[72]Bodansky O The Accelerant Effects of L Amino Acids on the Activity of Bone Phosphatase *J Biol Chem 114 273 (1936)*

[73]Bodansky O The Mechanism of Inhibition of Phosphatase Activity on Glycine *J Biol Chem* 165 605 (1946)

[74]Bodansky O The Inhibitory Effects of DL Alanine L Glutamic Acid L Lysine and L Histidine on the Activity of Intestinal Bone and Kidney Phosphatase *J Biol Chem* 174 465 (1948)

[75]*Bodansky O The Influence of Magnesium and Cobalt on the Inhibition of* Phosphatases of Bone Intestine and Osteogenic Sarcoma by Amino Acids *J Biol Chem* 179 81 (1949)

[76]Klemperer F W Miller J M and Hill C J Inhibition of Alkaline Phosphatase by Beryllium *J Biol Chem* 180 281 (1949)

[77]Grier, R. S Hood M B and Hoagland M B Observation on the Effects of Beryllium on Alkaline Phosphatase *J Biol Chem* 180 289 (1949)

[78]Yu T F and Gutman A B Effect of Beryllium on *In Vitro* Calcification of Cartilage *Proc Soc Exper Biol and Med* 75 481 (1950)

Dr Gutman We have found that concentrations of 1×10^{-4} M or 1×10^{-5} M beryllium exert no demonstrable inhibitory effect on calcification in the presence of inorganic phosphorus although calcification is completely inhibited when the beryllium concentration is increased to 1×10^{-3} M With beta glycerophosphate as the sole source of phosphorus inhibition is complete at the lower concentrations (1×10^{-5} or 1×10^{-4} M) of the inhibitor However we have noted that the effect of beryllium on certain phosphoric esters in the glycolytic cycle (glucose 1 phosphate or hexose di phosphate) could not be distinguished from its effect on beta glycerophosphate (Table XI)

Results with L-histidine

When 1×10^{-2} M L histidine was added to the incubating solution a similar phenomenon was observed Calcification in the presence of inorganic phosphate was not affected by this concentration of the amino acid although striking inhibition was observed when glucose 1 phosphate hexose di phosphate beta glycerophosphate or phenylphosphate was used as the sole source of phosphorus (Table XII) (In many of the bone slices in which inhibition was observed a thin amorphous line of silver deposition was observed in the region where calcification would have ordinarily occurred in the absence of the inhibitor This bore no resemblance to the characteristic orderly intercellular pattern of calcification usually seen) [9] Bodansky s demonstration[74] of a greater inhibitory effect on bone phosphatase of L histidine as compared with other a amino acids was borne out in our experiments with glycine (Table XIII) This amino acid in 1×10^{-2} M concentration had no discernible effect on calcification in the presence of glucose 1 phosphate or beta glycerophosphate

The Role of Phosphorylative Glycolysis in Calcification

These results might imply that phosphoric esters must undergo dephosphorylation before entry into the cell However evidence to the contrary comes from the demonstration[80, 81] that calcification will proceed after the glycolytic cycle has been blocked if a phosphoric ester in the cycle below the level blocked is supplied but will not in the presence of inorganic phosphorus alone

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[9] Experiments carried out since this report was given have shown complete disappearance of this amorphous deposit with higher concentrations of histidine

[80] Gutman A B and Yu T F Further Studies of the Relation Between Glycogenolysis and Calcification in Cartilage *Trans Macy Conference on Metabolic Interrelations* 1 11 (1949)

[81] Gutman A B and Yu T F A Concept of the Role of Enzymes in Endochondral Calcification *Trans Macy Conference on Metabolic Interrelations* 2 167 (1950)

TABLE XI

Effect on Calcification of Beryllium in Presence of Various Sources of P

Degree of Calcification	Source of Phosphorus											
	Inorganic P (5 mg %)			Glucose 1 Phosphate*			Hexose di Phosphate*			β-glycero-phosphate*		
	Control	Be 10^{-5} M	Be 10^{-4} M	Control	Be 10^{-4} M	Be 10^{-6} M	Control	Be 10^{-4} M	Be 10^{-5} M	Control	Be 10^{-4} M	Be 10^{-6} M
4+	7	—	5	6	—	—	—	—	—	12	—	—
3+	1	—	1	2	—	—	5	—	—	—	—	—
2+	—	—	2	—	—	2	—	—	—	1	—	4
1+	—	2	—	—	—	2	—	—	—	—	—	—
0	—	4	—	—	8	—	—	5	5	—	13	—
Number Bone Slices	8	6	8	8	8	4	5	5	5	13	13	4

*In concentration equivalent to 10 mg % inorganic phosphorus

TABLE XII

Effect on Calcification of L Histidine 1×10^{-2} M in Presence of Various Sources of P

Degree of Calcification	Source of Phosphorus: Inorganic Phosphate (5 mg %)		Glucose 1 Phosphate*		Hexose di Phosphate*		β Glycero Phosphate*		Phenyl Phosphate*	
	Control	Histidine	Control	Histidine	Control	Histidine	Control	Histidine	Control	Histidine
4+	15	13	8	—	5	—	10	—	4	—
3+	2	4	1	2	3	—	1	—	2	—
2+	—	—	2	3	1	—	1	—	—	—
1+	—	—	1	1	1	2	—	—	—	1
0	—	—	—	6	—	8	—	12	—	5
Number Bone Slices	17	17	12	12	10	10	12	12	6	6

*In concentration equivalent to 10 mg % inorganic phosphorus

TABLE XIII

Effect on Calcification of Glycine 1×10^{-2} M in Presence of Ester Phosphate

Degree of Calcification	Source of P			
	Glucose 1 Phosphate*		β Glycero Phosphate*	
	Control	Glycine	Control	Glycine
4+	4	3	3	4
3+	—	—	1	—
2+	—	—	—	—
1+	—	1	—	—
0	—	—	—	—
Number Bone Slices	4	4	4	4

*In concentration equivalent to 10 mg % inorganic phosphorus

The demonstration of inhibition of calcification through interference ...th phosphorylative glycolysis and subsequent studies have led to the ...stulate that inorganic phosphorus reaches the bone matrix via the gly...lytic cycle and that the function of the latter system is to achieve a local ...rease in the concentration of phosphorus If such were the case inor...nic phosphorus ultimately deposited as bone salt would at sometime ...ring the process of calcification exist as glucose 1 phosphate and hexose ...phosphate Hence an inhibitor which exerts its action in the presence of ...se esters should have at least an equivalent effect in the presence of ...organic phosphate We have not found this to be true in the case of ...histidine 1×10 M or beryllium 1×10^{-4} M These results suggest ...t phosphorylative glycolysis may be essential in the process of endochon...l calcification *in vitro* for some purpose other than that of building up ...ocal concentration of phosphorus The question has been raised as to ...ether glycolysis may function to produce energy for calcification al...ugh there is no information available concerning the energy require...nts of this process While our data obviously cannot be considered to ...vide specific support of such a possibility they are compatible with it

Relation of Inhibitors to Alkaline Phosphatase

The second question with which we are concerned that is whether the

inhibitors we have employed exert their adverse effect on calcification by means of an action on alkaline phosphatase arose as a result of inorganic phosphorus determinations carried out after incubation of bone slices for 18 hours in media that initially contained ester phosphate as the sole source of phosphorus We have found that the amount of inorganic phosphorus after 18 hours of incubation in media containing beryllium 1×10^{-4} M or L histidine 1×10^{-2} M was not significantly less than that present in the absence of the inhibitor (Figure 30) Our efforts to explain this phenomenon led to an investigation of factors which might raise the concentration of inorganic phosphorus in solution and which might thereby mask an inhibition of alkaline phosphatase activity

The phosphate esters employed were found to be most stable in solution for 18 hours and hence the possibility of spontaneous hydrolysis was ruled out (Table XIV) The inhibitors could not be implicated as contributing to the hydrolysis of the ester for incubation of ester phosphate with L histidine 1×10^{-2} M or beryllium 1×10^{-4} M led to no significant release of inorganic phosphorus (Table XIV) The extent to which phosphate diffused outward from the bone slice into the medium was determined by incubating bone slices in solutions devoid of inorganic phosphorus Since

TABLE XIV

Possible Sources of Inorganic Phosphorus in the Incubating Medium

Bone Slice	Source of P		
	Hexose Di Phosphate (10 mg % P)	0 01 M Histidine	Inorganic P After 18 Hours Incubation
+	+	0	*5 5 mg /100 cc
+	+	+**	*6 3 mg /100 cc
0	+	0	*0 4 mg /100 cc
0	+	+**	0 0 mg 100 cc
+	0	0	0 3 mg 100 cc
+	0	+**	*0 3 mg /100 cc

Incubating medium contained in addition basal solution and calcium 9 6 mg %

* Average of three or more experiments

**Similar experiments using Be 1×10^{-4} M as inhibitor gave comparable results

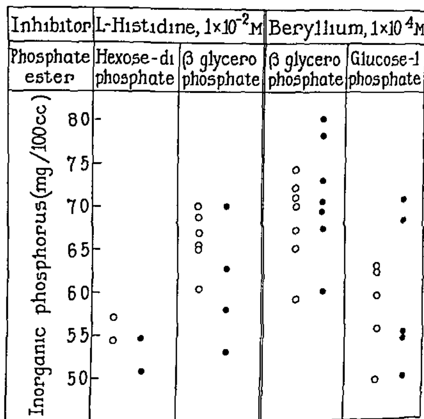


Fig 30 Effect of L Histidine and Beryllium on Phosphorus Liberation from Phosphate Esters after Incubation with Rachitic Bone Slice

The phosphate esters were calculated to yield 10 mg % P on complete hydrolysis The incubation was continued for 18 hours at 37 C

after 18 hours the concentration of inorganic phosphorus did not exceed 0 3 mg/100 ml (Table XIV) one can assume that in our experiments ester phosphate was the only significant source of phosphorus present in the incubating medium It also seemed possible that despite the comparable inorganic phosphorus levels after 18 hours of incubation there had been a delay in the release of inorganic phosphorus in the presence of the inhibitor If such were the case one might postulate that by the time sufficient inorganic phosphorus were present to permit bone salt deposition, changes might occur in the bone slice (e g loss of glycogen) which would prevent calcification from taking place Using beryllium 1×10^{-4} M as inhibitor and doing serial determinations of inorganic phosphorus beginning at one hour of incubation we were able to discern no significant lag as compared with incubation in the absence of the inhibitor (Figure 31) [Similar experiments with L-histidine 1×10^{-2} M as inhibitor in progress at the time this report was given revealed a definite delay in the liberation of inorganic phosphorus (Figure 32) Using higher concentrations of L histidine ((3×10^{-2} M)) we have found the level of inorganic phosphorus *after 18 hours of incubation to be reduced by 50%] It should be stressed* that although our method involves the use of one face of the bone slice in the control solution while its opposite is placed in the test solution we *cannot possibly insure that each bone slice contains identical amounts of* alkaline phosphatase or even of epiphyseal cartilage This short coming might conceivably cast some doubt on the validity of these experiments, but one should expect to be able to detect differences in the amounts of inorganic phosphorus released if the inhibition by beryllium 1×10^{-4} M in the cartilage slice approached the 80% inhibition demonstrated by Dr Gutman in cartilage homogenates

The possibility that beryllium exerts its adverse action on calcification by some mechanism other than that of inhibition of alkaline phosphatase will be explored

Summary

1) Endochondral calcification of rachitic rat epiphyseal cartilage *in vitro* can be inhibited by beryllium 1×10^{-4} M or L histidine 1×10^{-2} M, when phosphate ester is present as the sole source of phosphorus Much higher concentrations of these inhibitors are required for inhibition of calcification when inorganic phosphate is provided in the incubating medium It is at present difficult to reconcile this finding with the concept that the function of phosphorylative glycolysis in endochondral calcification is to achieve a local increase in phosphorus concentration

2) Despite their marked inhibitory effect on endochondral calcification *in vitro* beryllium, 1×10^{-4} M and L histidine 1×10^{-2} M, effect no

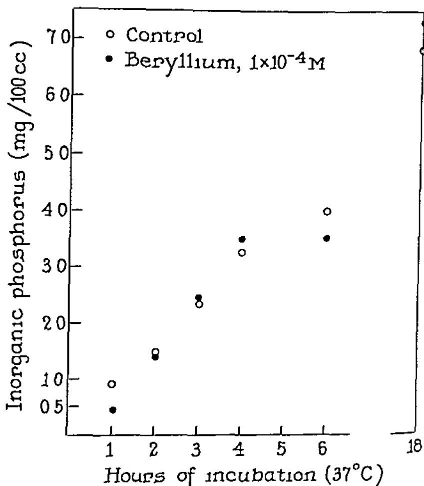


Fig 31 Effect of Beryllium on the Rate of Inorganic Phosphorus Liberation from β glycerophosphate by the Rachitic Bone Slice

The concentration of beryllium was 1×10^{-4} M The β glycerophosphate was calculated to yield 10 mg % P on complete hydrolysis

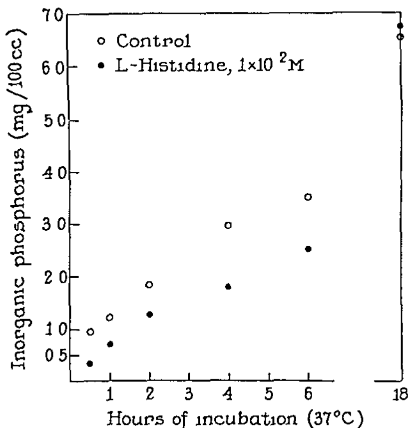


Fig 32 I ffect of L H st d ne on tl e Rate of Inorgan c Pl osj hori s L l erat o fron β gl ceropl ospl ate l the Rach t c Bone Sl ce

The co centra on of L-H st d ne as 1 × 10⁻² M The β glycerophospha e as calcu ated t y eld 10 mg % I on compl te l yd o y s

significant reduction of ester phosphate hydrolysis after 18 hours of incubation Higher concentrations of L histidine lead to a considerable diminution in ester phosphate hydrolysis The possibility that calcification is inhibited by an action of beryllium other than that of interference with dephosphorylation by alkaline phosphatase must be considered

Conference Discussion

Neuman The ester phosphate was hydrolyzed?

Hiatt The ester phosphate in the medium containing beryllium was hydrolyzed to the same extent as it was in the control solution without inhibitor We have considered one additional possibility one which seems quite unlikely but which we shall investigate Is it possible that there is a significant amount of phosphorus removed from solution and deposited as bone salt in the media in which calcification proceeds? If such were the case similar levels of phosphorus in the control and in the beryllium containing media would indicate that more ester had been hydrolyzed in the former

We have done some preliminary experiments to determine whether the effect of the inhibitor is discernible after short periods of contact with the bone slice We have noted that a bone slice in contact with beryllium 1×10^{-4} M for one hour and then washed in basal solution several times will not calcify in a medium containing basal solution calcium and ester phosphate

Hastings How far can you run that?

Hiatt Thus far we have run this experiment with only one concentration of beryllium 1×10^{-4} M

Armstrong That is very interesting Have you any comment to make on this Dr *Shorr*?

Shorr We need a unifying concept which we do not have in order to explain all these discrepancies in each of the cycles Some of the experiments which Dr Hiatt is projecting relate to the reincubation in the medium after 18 hours of incubation when the concentrations of phosphate have risen to levels which should deposit that is utilizing that medium as the medium for fresh bone slices So far you are not willing to discuss your results?

Hiatt That is right

Shorr I should appreciate your comments Dr Neuman on these alterations in the phosphate content of the medium despite what seems to be a situation which is not favorable for the splitting of β glycerophosphate

Neuman I cannot explain that one

Did you also find a corresponding decrease in the amount of ester phosphate in solution or did you not determine that?

Hiatt We did not determine that

Neuman It is a little difficult to determine whether phosphate has dissolved from preformed calcification which does exist in these slices, or whether this represents truly the hydrolysis of ester phosphate It could be determined at least in a rough fashion

Shorr Dr Hiatt has control observations which rather uniformly show that only a very minimal amount of phosphate might have come from bone about a half milligram in 18 hours

Neuman I would be very much surprised if it were as high as he observed I believe one could show that the ester concentration had been decreased All I can think of is that the beryllium is being bound by the ester and that one is not getting effective inhibition of the phosphatase even though in the powder experiments inhibition is more or less complete

Gutman In our *in vitro* calcification experiments we did not check the inorganic phosphate split off by our slices from β glycerophosphate into the incubating fluid in the presence and absence of beryllium as Dr Hiatt did However we have similar data in connection with our time activity curves indicating the inhibition of cartilage *homogenate* alkaline phosphatase by beryllium salts in various concentration These show striking differences at the end of 1 hour about 35 γ inorganic phosphate in the control solutions without beryllium and 30 γ split off in the presence of $1\ 0 \times 10^{-6}$ M Be but only 10 12 γ inorganic phosphate split off in the presence of $1\ 0 \times 10^{-5}$ M $1\ 0 \times 10^{-4}$ M and $1\ 0 \times 10^{-3}$ M Be Dr Hiatt I understood you to say that when you incubate whole cartilage slices there was no difference in inorganic phosphate liberated in the presence or absence of beryllium salts in concentrations of 10^{-4} M

Hiatt That is right Dr Gutman On occasion we have demonstrated differences of 0 3 or 0 4 mg per cent but none that we have considered significant

Gutman Of course it should be kept in mind that our cartilage homogenate studies were short term experiments conducted at pH 9 6 near the optimum for alkaline phosphatase whereas *in vitro* calcification of whole cartilage slices involves many hours and the incubating medium is adjusted to pH 7 4 at which dephosphorylation by alkaline phosphatase is slow and relatively feeble

McLean Dr Gutman the differences you found with cartilage homogenate were apparent at the end of one hour?

Gutman As our time activity curves show, the difference was apparent in the first five minutes but the figures I cited were those at the end of one hour

Follis Would you comment on the present status of beryllium rickets?

Gutman The action of beryllium in producing 'beryllium rickets was originally thought to depend entirely upon the removal of phosphate from the gastrointestinal tract by precipitation as beryllium phosphate, with corresponding lowering of serum inorganic phosphate levels However, as Dr Sobel and others have shown, there is considerable evidence that beryllium also has a deleterious effect on the local factor of calcification The inhibition of alkaline phosphatase and interference with *in vitro* calcification of cartilage more recently described support the contention that beryllium acts adversely upon the local mechanisms of endochondral calcification However, since this effect would depend on the concentration of beryllium at the site of calcification, and since this concentration is not known in beryllium feeding experiments, I cannot say how significant the local action of beryllium might be in the production of "beryllium rickets'

Follis Does anybody know whether the serum concentration of phosphatase is any different in animals that have gotten beryllium?

McLean Yes

Follis Is it lowered?

McLean Yes

Urist Does beryllium actually come down in the bone ash?

Neuman Yes

Urist In beryllium rickets?

Neuman Yes that is right, too

Hodge In very small amounts

Neuman The blood levels for beryllium that can be obtained even on injection of a soluble salt, are extremely minute, a few millimicrograms per ml

Copp And the absorption from the intestinal tract is very small so it is unlikely that it would be a dominant factor

Neuman But whether or not there could be a local concentration still is a possibility

Gutman It would have to be of the order of 10^{-3} M

Neuman Locally?

Gutman Yes

Neuman That could happen I certainly could not deny that possibility

Sobel I figure this out a little bit differently Calcification *in vitro* with organic phosphate takes place due to the release of inorganic phosphate providing the Ca $\times$ P product necessary for calcification during the course of incubation Following this line of reasoning one can state that beryllium ions directly inactivate the calcifying mechanism[82] so that the higher the beryllium concentration, the higher Ca $\times$ P product one needs in order to produce calcification Moreover, the higher the organic phosphate, the higher the ultimate inorganic phosphate in the system with a given amount of beryllium

My postulate, as you know is that beryllium combines also with something else than the enzymes involved in handling organic phosphates, in producing inhibition of the calcifying mechanism It competes with calcium for something in the cell which is essential for calcification

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[82] Sobel, A. E. The Local Factor in Calcification *Trans Macy Conference on Metabolic Interrelations* 2 113 (1950)

SUMMARY ON OSTEOGENESIS

FRANKLIN C McLEAN

From the Department of Physiology Division of Biological Sciences the University of Chicago Chicago Ill

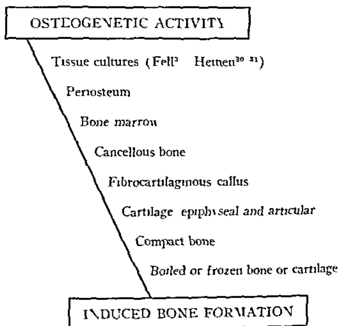
Armstrong I will ask Dr McLean if he will be good enough to summarize the presentation and discussion having to do with osteogenesis

McLean I promised to be brief so I shall confine myself to one indication of what we think we have learned from the experiments that Dr Urist presented yesterday

I have arranged the tissues studied by Dr Urist in the form of a scale as shown in Table XV At the upper end of the scale we have cells that

TABLE XV

Classification of the Relative Activity of Tissues in Producing Bone



are clearly capable of making bone by virtue of their own osteogenetic activity At the lower end of the scale it is necessary to assume that bone is being formed by the cells of the host under the influence of an inductor

transplanted with devitalized tissues In the middle of the scale it seems likely that both factors are operating some cells from the implant producing bone while the implanted material may also be exercising an influence on the cells of the host inducing them to produce bone

Dr Armstrong has asked why it is necessary to bring in the concept of the inductor at all I think that can be answered very simply when you implant boiled or frozen bone or cartilage and new bone results certainly this has to be thought of as induced by the transplanted devitalized material To a lesser extent the concept seems applicable for other tissues that may still be living but that act as inductors

Fibrocartilaginous callus acts in such a way as to suggest that it may both produce new bone from its own cells and also induce bone formation by the cells of the host The transplants of fibrocartilaginous callus in the eye act very much as does the fibrocartilaginous callus in a healing fracture In both cases it is prone to replacement by bone which grows into the callus from the periphery but it shows less evidence of being able to produce bone from its own cells However we cannot rule out the possibility that some of the cells in the fibrocartilaginous callus when implanted in the eye actually produce bone themselves

Cartilage—epiphyseal or articular—certainly rates low in the scale It seems very unlikely that bone is produced by the transformation of these cartilage cells into bone It is much more likely that the living cartilage behaves in a fashion similar to that of the boiled cartilage in both cases the cells of the recipient being induced to form bone

Compact bone free from marrow and stripped of periosteum certainly does not produce bone from its own cells to any great extent This is particularly important with respect to the bone banks that are coming into common use at the present time We have not discussed the question of bone banks at any length but our evidence supports the view that grafts of compact bone even when fresh and autogenous produce little or no bone from their own cells but that they do serve as inductors leading to formation of bone by the cells of the host On the other hand grafts of cancellous bone presumably because of their high content of other connective tissue elements including endosteum and the cells of the bone marrow appear to have greater ability to produce new bone from their own cells

Conference Discussion

Shorr One of the tests for determining inductor activity was the capacity of boiled bone to induce bone growth I wonder whether that same test has been explored up and down the scale whether a boiled peri

osteum or a boiled tissue culture has been explored and found to lose or retain its osteogenetic activity

Urist We do not have the information about periosteum devitalized by boiling However DeBruyn observed that 12 samples of frozen periosteum did not produce bone formation

McLean You say it will induce it?

Urist No it did not produce bone

Shorr It is a little less certain than the boiled treatment isn t it so far as cell viability is concerned?

Urist How vulnerable it is in periosteum that is relatively speaking I do not know

Shorr But you have demonstrated the thermostability of the inductor factor

Urist Yes I think it is thermostable

Shorr So that might be a good criterion to use

Urist We have first hand information on cartilage both frozen and boiled and we know that the inductor will resist such treatment

Howard May I ask Dr McLean a question? It goes back to the old idea—I think it has been discarded frequently in these meetings—that if you put calcium in high concentration next to a fibroblast it may become interested in making bone Is calcium an inductor as you call it? Have you put any apatite salts into say the tendons of the rectus sheath?

McLean Heinen[83] did it in his rats

Howard Bone powder does it that is makes bone formation

As you go down the scale here—and your cells are actual osteoblasts already aren t they—it looks as though maybe the amount of calcium in the transplanted tissues has something to do with its bone stimulating properties

McLean Heinen used it in rabbits He used calcium chloride which is of course quite irritating

Howard That is a terrible stunt!

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[83] Heinen J H Jr Dabbs G H and Mason, H A The Experimental Production of Ectopic Cartilage and Bone in the Muscles of Rabbits *J Bone and Joint Surg* 31 765 (1949)

McLean With calcium chloride, he induced bone formation in rabbits We have the feeling that almost any irritating substance will induce bone formation in the rabbit's muscle I do not think that answers your question very directly

Howard There is one other thing that has always bothered me which you have not mentioned as an inducer or stimulant to osteogenetic activity It is probably in your paper I refer to the experiments of Pearse and Morton[84] wherein you tie off the vein and apparently induce increased venous pressure, increased carbon dioxide tension or whatever you do under those circumstances There is little doubt that they hastened the healing of fractures in the dog

You have not mentioned that as a stimulant

McLean No and I have not mentioned the production of bone in the kidney by a similar procedure

Urist I omitted to mention an experiment in which we put some bladder mucosa in the eye and did not get bone within 30 days I do not know whether, if we had made a sandwich of bladder plus fascia and put that in the eye, it would have made bone but the bladder mucosa alone — as a homogenous transplant — did not do it in 30 days

Follis Will the bladder mucosa work elsewhere?

Urist The bladder mucosa will do it if you lay it on fascia

Follis In the rat?

Urist I do not know whether it has been done in the rat

Robinson Huggins[85] found that bone forms adjacent to proliferating epithelium of transplanted autogenous bladder "in the connective tissues of the fatty-fibrous subcutaneous tissue, of striated muscle of the fasciae (rectus sheath, fascia lata fascia covering the sacrospinalis muscle) and capsule of the knee joint in the dog and the fibrous tissue that forms beneath the proliferating pelvic epithelium after ligation of the renal vessels in the rabbit" Huggins also found that bone did not form when bladder epithelium as an autogenous transplant was placed in the "fibromuscular wall of the urinary bladder and connective tissue stroma surrounding trans plants of mucosa of the bladder in the liver spleen and kidney *in the dog* and the fascia of the rectus sheath and the fascia lata *in the rabbit*"

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[84] Pearse H E Jr and Morton J J The Stimulation of Bone Growth by Venous Stasis *J Bone & Joint Surg* 12 97 (1930)

[85] Huggins C B The Formation of Bone Under the Influence of Epithelium of the Urinary Tract, *Arch Surg* 22 377 (1931)

Urist As to Dr Howard s question about calcium salt, I think there is some answer for that It needs a little more work

Bisgard[96] put some calcium salt in the eye (calcium phosphate) and got no bone The experiment was intended to test the thesis of Leriche and Policard[97] that 'calcium plus fibroblasts' equals bone

Does that answer your question?

Howard I don't think so

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[96]Bisgard J D *Ossification the Influence of Mineral Constitutents of Bone Arch Surg* 33 926 939 (1936)

[97]Leriche R and Policard A *The Normal and Pathological Physiology of Bone* Transl by A J Key C V Mosby & Co, St Louis (1928)

EFFECTS OF PITUITARY FACTORS AND OF THYROXIN ON SKELETAL MORPHOGENESIS IN THE RAT[88, 89]

C WILLET ASLING, MIRIAM E SIMPSON, and HERBERT M EVANS

From the Institute of Experimental Biology and the Division of Anatomy University of California Berkeley

Armstrong Gentlemen the program for the evening session will be presented by Dr Asling of the University of California

Asling The hormones of the anterior hypophysis may be divided into two main groups—those having primarily metabolic effects and those exerting their influence through the reproductive system Of the former group growth hormone and adrenocorticotropic hormone have been used in pure form for over six years[90, 91] The final purification of the thyrotropic hormone not yet having been accomplished its action must at present be inferred from the action of its target organ hormone thyroxin This presentation will be restricted to the influence of growth hormone ACTH and thyroxin on skeletal morphogenesis the gonadotropins and sex hormones will not be treated

Some Factors Involved in Endocrine Experiments

Before describing the actions of these hormones on the growth and maturation of the skeleton it is necessary to comment briefly on four factors

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[88]The au
here have
Robert I
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the U S Public Health Service and the American Foundation for Dental Science Other grants [illegible] have been acknowledged in reports cited in the bibliography
It should also be acknowledged that this presentation has drawn heavily for its organization and content from a recent summary by Simpson *et al*[89]

[89]Simpson M E, Asling C W and Evans H M Some Endocrine Influences on Skeletal Growth and Differentiation *Yale J Biol and Med* 23 1 27 (1950)

[90]Li C H Evans H M and Simpson M E Isolation and Properties of the Anterior Hypophyseal Growth Hormone *J Biol Chem* 159 353-366 (1945)

[91]Li C H Evans H M and Simpson M E Adrenocorticotropic Hormone, *J Biol Chem* 149 413-424 (1943)

involved in endocrine experiments, namely (1) purity of hormones, (2) nutritional conditions (3) sensitivity of techniques, and (4) any pertinent peculiarities inherent in the experimental animal

Purity of Hormones

The pituitary hormones used in our experiments have been pure in the sense that they were electrophoretically homogeneous and that on biological assay they showed no evidence of other hormonal contaminants when injected at many multiples of the minimal effective dose Such conditions are particularly important in disclosing the separate roles of the hormones under study here, since growth hormone and thyrotropic hormone[92] are known to be synergistic and on the other hand ACTH opposes the action of growth hormone on body growth[93] The presence of one of these as a contaminant in another might prevent clear demonstration of the action of the hormone under primary study

Nutritional Conditions

The diets employed must be adequate in all known essentials In our experiments a modified McCollum's diet (Diet I) is given (usually as a wet mash) to all rats in endocrine deficiency states This diet appears optimal for growth reproduction, and lactation Hypophysectomized rats have been maintained on it in considerable numbers for over two years and occasionally for as long as three years

It is important to determine to what extent altered appetite and food consumption contribute to stimulation or failure of growth and also to take account of specific increase in nutritional requirements induced by hormonal therapy The increased requirement of the B vitamins when thyroxin is administered is an example of the latter Also to be considered is the probability that differing hormonal regimens may affect differently the intestinal absorption of the food ingested

Sensitivity of Techniques

Body weight is the simplest measure of growth However, since chemical analysis would be necessary to establish that the weight increase represents

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[92] Marx W Simpson M E and Evans H M Synergism Between Thyrotropic and Growth Hormones of Pituitary Body Weight Increase in Hypophysectomized Rat *Proc Soc Exp Biol and Med* 49 594 597 (1942)

[93] Marx W Simpson M E Li C H and Evans H M Antagonism of Pituitary Adrenocorticotropic Hormone to Growth Hormone in Hypophysectomized Rats *Endocrinol* 33 102 105 (1943)

true growth rather than accumulation of fat, measurements of length are also essential. The tail length can be measured without anaesthesia, and the total body length under anaesthesia. Individual bones may be measured at autopsy and under certain conditions in roentgenograms. If serial roentgenograms have been prepared during an experiment, the growth of the bone may thus be followed. The error in these linear measurements has not exceeded two parts in a hundred.

Diagnosis of the status of epiphyses in roentgenograms of small animals requires a fine-grain film. Reasonable emulsion speed is necessary, especially when working with living animals. Eastman Industrial Type A X-ray film has adequate speed, and the grain is sufficiently small that it is not objectionable when studied at five to ten times magnification.

The Rat as an Experimental Animal

The advantages of using a small animal (cage space, dosage of hormones, etc.) are obvious. The very substantial body of knowledge respecting nutritional requirements of rats also is advantageous. However, rats are often criticized as unsuitable for experiments involving growth and maturation. One concise expression of a common opinion states that ". . . epiphyseal union, even in the extremities, is delayed into extreme old age and may never be completed. This appears to be a truly primitive condition, reminiscent of the reptiles in which growth may continue into old age. It is unfortunate that the animals most commonly used in the study of growth and aging possess the primitive characters of delayed union and long continued growth, which contrast so markedly with the sharply defined period of growth characteristic of the vast majority of placental mammals."[94]

In the face of this opinion, it is necessary to investigate briefly some of the phenomena of skeletal morphogenesis in normal rats of the strain used in experiments reported here (Long-Evans). Figure 33 shows the total body length of normal female rats from infancy to two years of age. The difference between the lengths at 250 days and at 720 days was 5 mm, and did not prove significant. The length of the tibia of adult normal female rats (Figure 34) also does not increase. It has been shown that at the inflection point of the growth curve of this bone (60-90 days of age) its proximal epiphyseal plate undergoes an abrupt narrowing.[95] The plate re-

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[94] Washburn, S. L. The Sequence of Epiphyseal Union in the Opossum. *Anat. Rec.* 95:353-363 (1946).

[95] Becks, H., Simpson, M. E., and Evans, H. M. Ossification at the Proximal Tibial Epiphysis in the Rat. I. Changes in Females with Increasing Age. *Anat. Rec.* 92:109-119 (1945).

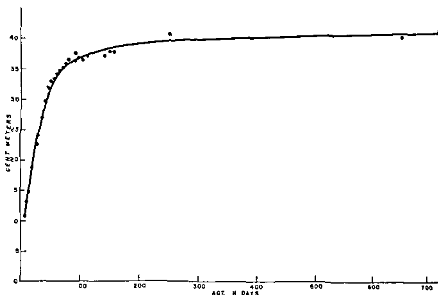


Fig 33 Curve Showing Growth in Total Body Length of Normal Female Rats

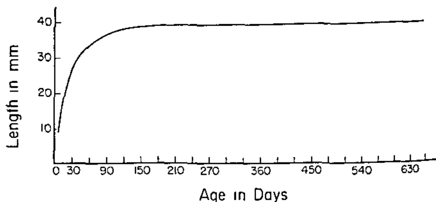


Fig 34 Curve Showing Growth of Tibia of Normal Female Rats

After Simpson *et al*[89]

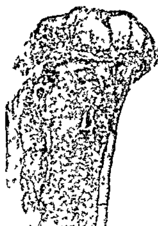








Fig 35 Epiphyseal Closure in the Rat Tibia

A Proximal tibial epiphysis of normal female rat showing complete epiphyseal union (H & E stain × 10) Age 720 days unusually early union *B* Distal tibial epiphysis of normal female rat (Mallory stain × 10) Age 11 days epiphyseal plate intact *C* Distal tibial epiphysis of normal female rat (Mallory stain × 10) Age 91 days epiphyseal plate perforated *D* Distal tibial epiphysis of normal female rat (Mallory stain × 10) Age 101 days epiphyseal plate absent

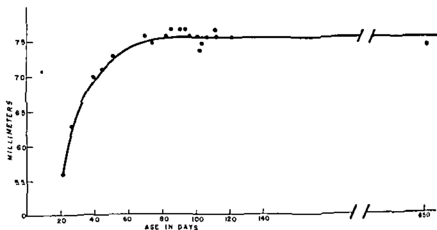


Fig 36 Curve Showing Growth of Metacarpal of Normal Female Rats

mains however, and eventually (starting at approximately 170 days of age) becomes sealed from the diaphyseal marrow cavity by a transverse lamina of bone[96] After this, the growth of the bone ceases Areas of cartilage erosion may persist Dawson[97] gave the age of epiphyseal closure as 1,135 days Figure 35A shows very early closure in this bone (720 days) During the period when the proximal epiphyseal plate is narrowing, the distal epiphyseal plate is undergoing removal (Figure 35 B C D) and fusion is completed before 100 days of age

Some of the epiphyses of other long bones also show epiphyseal fusion The metacarpal is of particular interest Its single distal epiphyseal center fuses at approximately 100 days of age[98] Thus the activity at only one epiphysis accounts for growth of this bone As seen in Figure 36 the definitive length of the bone is attained 20 30 days before its epiphyseal union occurs

The distal end of the humerus has been examined and found to have a more complex ossification pattern[99] The sequence of events up to 40 days of age appears in Table XVI and is quite constant After its main epiphyseal ossification center fuses with the diaphysis activity is sustained at the separate epiphysis in the medial epicondyle where fusion does not occur until approximately 120 days of age Histological studies of this bone thus become useful in investigations on skeletal maturation over a broad span of chronological ages

Thus our experiments have been conducted on an animal form which undergoes no significant degree of continued growth and in which at least some of the epiphyses show *closure in early adult life In fact numerically* the majority of epiphyseal cartilage plates may disappear Those epiphyses which remain patent are in the minority They occur notably in the limbs and tail Each of the major long bones has one epiphysis which remains patent into senescence In the ulna and the femur both epiphyseal discs remain Patency of the epiphyses of caudal vertebrae has also been recognized histologically even in old age[89]

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[96] It is interesting that this lamina is clearly defined as the line of growth arrest in roentgenograms

[97] Dawson, A B The Age Order of Epiphyseal Union in the Long Bones of the Albino Rat *Anat Rec* 31 1 17 (1925)

[98] Becks H Asling C W Collins D A Simpson M E and Evans H M Changes with Increasing Age in the Ossification of the Third Metacarpal of the Female Rat *Anat Rec* 100 577 592 (1948)

[99] Becks H Asling C W Simpson, M E Evans H M and Li C H Ossification at the Distal End of the Humerus in the Female Rat *Am J Anat* 82 203 230 (1948)

TABLE XVI

Progressive Stages in the Development of the Distal End of the Humerus in Normal Female Rats between 10 and 45 Days of Age

Age in Days	Ossification Center	
	Main (Capitulum Trochlea)	Medial Epicondyle
10	Cartilaginous	Cartilaginous
15	Cartilaginous central masses of calcified matrix and enlarged vacuolated cells foreshadow ossification center	Cartilaginous
20	Ossification center active expanded cartilage plate established often already perforated at one point	Cartilaginous central masses of calcified matrix and enlarged, vacuolated cells foreshadow ossification center
25	Cartilage plate further interrupted reduced to several bars of cartilage	Ossification center active expanded cartilage plate established
30	Small fragments of cartilage except for substantial bar in *lateral* epicondyle	No further change
35	Narrow cartilage bar in *lateral* epicondyle only	No further change
40	No cartilage remaining epiphyseal fusion complete	No further change
45	No further change	No further change

Adapted from the report of Becks *et al*[99]

By study of roentgenograms of normal rats of increasing ages a table of the times of appearance of ossification centers and of their fusion may be constructed Table XVII shows such a table in condensed form When more detailed such a table may be used to determine the "skeletal age" of any animal at any time in its life

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[100]Scow R O Simpson [illegible] Asling [illegible] C H and Evans H M [illegible] Response by the Rat [illegible] ro de [illegible] irth to Gr wth Hormone and to Thyroxin Given [illegible] in Cr [illegible] General Growth and Organ Changes *Anat Rec* [illegible] 9)

[101]Ray R. D , Simp [illegible] H [illegible] and Evans H M Effects of the [illegible] y Gro [illegible] th and Differentiation of the [illegible] *nat* 86 479 516 (1950)

TABLE XVII

Determination of Skeletal Ages

Ossification Center	Chronological Age in Days															
	12	15	18	21	25	30	40	50	60	80	100	120	Latent Period	940	1006-1091	1135+
Humerus proximal	P	—	—	—	—	—	—	—	—	—	—	—		—	—	A
distal	P	—	—	—	—	—	A									
medial epicondyle				P	—	—	—	—	—	—	—	A				
Radius proximal		P	—	—	—	—	—	—	—	A						
distal	P	—	—	—	—	—	—	—	—	—	—	—		—	—	A
Ulna proximal		P	—	—	—	—	—	—	—	—	—	—		A		
distal	P	—	—	—	—	—	—	—	—	—	—	—		—	—	A
Metacarpal distal	P	—	—	—	—	—	—	—	—	—	A					
Phalanges		PI	—	PII	—	—	—	—	—	—	A					
row I II III				PIII												
Femur proximal								P	—	—	—	—		—	A	
trochanter					I	—	—	—	—	—	—	—		—	A	
distal	P	—	—	—	—	—	—	—	—	—	—	—		—	—	A
Patella						P										
Tibia proximal	P	—	—	—	—	—	—	—	—	—	—	—		—	—	A
distal	P	—	—	—	—	—	—	—	—	—	A					
Metatarsal distal	P3	P2	P4	P1	—	—	—	—	—	—	A					
				P5												
Pelvis acetabulum lip							P	A								
triradiate								A								

P represents present A represents absent

From Simpson *et al*[9] adapted from Scow *et al*[100] Ray *et al*[101] and Dawson[97]

TABLE XVI

Progressive Stages in the Development of the Distal End of the Humerus in Normal Female Rats between 10 and 45 Days of Age

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	Main (Capitulum Trochlea)	Medial Epicondyle
10	Cartilaginous	Cartilaginous
15	Cartilaginous central masses of calcified matrix and enlarged, vacuolated cells foreshadow ossification center	Cartilaginous
20	Ossification center active, expanded, cartilage plate established, often already perforated at one point	Cartilaginous, central masses of calcified matrix and enlarged, vacuolated cells foreshadow ossification center
25	Cartilage plate further interrupted reduced to several bars of cartilage	Ossification center active, expanded, cartilage plate established
30	Small fragments of cartilage except for substantial bar in *lateral* epicondyle	No further change
35	Narrow cartilage bar in *lateral* epicondyle only	No further change
40	No cartilage remaining, epiphyseal fusion complete	No further change
45	No further change	No further change

Adapted from the report of Becks *et al* [99]

By study of roentgenograms of normal rats of increasing ages a table of the times of appearance of ossification centers and of their fusion may be constructed Table XVII shows such a table in condensed form When more detailed such a table may be used to determine the "skeletal age" of any animal at any time in its life

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[100] Scow R O Simpson M E Asling C W, Li C H and Evans H M Response by the Rat Thyro parathyroidectomized at Birth to Growth Hormone and to Thyroxin Given Separately or in Combination I General Growth and Organ Changes *Anat Rec* 104 445-463 (1949)

[101] Ray, R. D , Simpson M E , Li C H , Asling C W , and Evans H M Effects of the Pituitary Growth Hormone and of Thyroxin on Growth and Differentiation of the Skeleton of the Rat Thyroidectomized at Birth, *Am J Anat* 86 479 516 (1950)



Fig 37 Comparison of Experimental Hypophysectomy with a Case of Human Pituitary Dwarfism

A Costochondral junction of hypophysectomized 60-day-old female rat 37 days after operation (H & E stain ×60) After Ray *et al* *B* Costochondral junction from human male 38 years of age diagnosis Nanosomia Pituitaria After Erdheim [103]

Effects of Removal of Endocrine Glands on Skeletal Development

Hypophysectomy

The 28 day old female rat has been commonly used in studies of hypophysectomy and will therefore be first described All primary and most secondary ossification centers have appeared by this age After hypophysectomy growth in weight ceases almost at once, marked reduction of growth in length occurs and also is eventually arrested Epiphyseal cartilage plates narrow abruptly Thus marrow contact with the cartilage is reduced The underlying spongiosa is resorbed and bone is laid down at the cartilage margin At the proximal epiphyseal plate of the tibia and of other bones which like it remain open until late in life, the plate prematurely comes to resemble that in the senescent normal rat in that a layer of bone seals it from the marrow cavity [102] It is noteworthy that this sealing off process resembles closely that described by Erdheim in a case of human pituitary dwarfism [103] The resemblance at the costochondral junction is illustrated in Figure 37

At those epiphyses which fuse earlier (80 120 days) such as the distal end of the tibia metacarpal and medial humeral epicondyle cartilage atrophy and sealing lamina of bone also develop rapidly The epiphyses remain separate for at least one to two years This effect of hypophysectomy in disturbing skeletal maturation was first shown by Dandy and Reichert[105] in roentgenograms of hypophysectomized puppies

At the distal end of the humerus where fusion of the main epiphyseal center is in progress at 25 days and is complete by 40 days of age differentiation continues after hypophysectomy at 28 days and reached complete epiphyseal fusion [106] Therefore the full effect of hypophysectomy on differentiation does not develop immediately and fusion of centers whose fusion is imminent progresses Table XVIII shows the effect of hypophysectomy at 28 days on epiphyseal fusion

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[102]Becks H Simpson M E and Evans H M Ossification at the Proximal Tibial Epiphysis in the Rat II Changes in Females at Progressively Longer Intervals Following Hypophysectomy *Anat Rec* 92 121 133 (1945)

[103]Erdheim, J Nanosomia Pituitaria *Beitr path Anat* 62 302-377 (1916)

[104]Ray R D Asling C W Simpson M E and Evans, H M Effects of Thyroxin Injections on Growth and Differentiation of the Skeleton of Hypophysectomized Female Rats *Anat Rec* 107 253 264 (1950)

[105]Dandy W E and Reichert F L Studies on Experimental Hypophysectomy in Dogs III Somatic, Mental and Glandular Effects, *Bull Johns Hopkins Hosp* 62 122 155 (1938)

[106]This was also found to be the case in studies of the metacarpal after hypophysectomy at 75 days of age [107]



Fig 38 The Degree of Differentiation Attained by 60 Days of Age by the Distal End of the Humerus of Rats Hypophysectomized at Different Ages

(H & E stain × 10) *A* Rats hypophysectomized at 6 days of age The main epiphyseal plate has persisted Establishment of the medial epicondylar ossification center has not occurred *B* Rat hypophysectomized at 28 days of age Epiphyseo-
... the operation The plate
The main epiphyseal plate
that in the medial epicon

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[110] Asling C W, Walker D G Simpson M E and Evans H M Differences in the Skeletal Development Attained by 60 day old Female Rats Hypophysectomized at Ages Varying From 6 to 28 Days *Anat Rec* 106 555 570 (1950)

TABLE XVIII

Epiphyseal Status of Rats Hypophysectomized at 28 Days of Age After a Postoperative Period of 480 Days

Epiphyseal Center	Normal Rats Age of Epiphyseal Closure in Days	Hypophysectomized Rats Epiphyseal Status
Humerus distal	40	−
Radius proximal	85	+
Tibia distal	90	+
Metacarpal third	100	+
Humerus medial epicondyle	120	+
Ulna olecranon	940	+
Humerus proximal	1135	
Radius distal	1135	
Ulna distal	1135	
Fibula proximal	1135	
Tibia proximal	1135	

Modified from Evans et al [8]
Epiphyses separate are marked + those fused are mar

It was sought to check the differentiation of plate by hypophysectomy at younger ages D G [?] physectomizing rats at 6 days of age [109] Figure of this operation on maturation of the humerus hypophysectomized at 6 days of age showed u

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[107]Asling C W Becks H Simpson M E and E Thyroxin Injections on Growth and Epiphyseal Closure in Hypophysectomized Female Rats *Anat Rec* 101 23

[108]Evans H M Asling C W Simpson M E and Hypophysectomized Female Rats Following Chronic T Growth Hormone IV Skeletal Changes Difference Intact Rats *Growth* 13 191 206 (1949)

[109]Walker D G Simpson M E Asling C W Differentiation in the Rat Following Hypophysectomy 106 539 554 (1950)

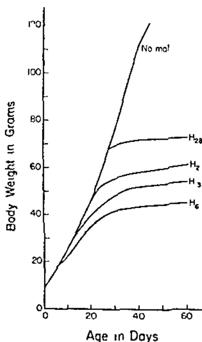


Fig 39 Curves Showing Gain in Body Weights of Rats Hypophysectomized at 6 13 21 or 28 Days of Age
After Walker *et al*[109]

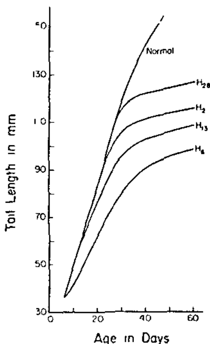


Fig 40 Curves Showing Increase in Length of the Tail of Rats Hypophysectomized at 6 13 21 or 28 Days of Age
After Walker *et al*[109]

not only in retention of a large portion of the main plate but also in the failure to appear of the medial epicondylar ossification center (Figure 38 A) The rat hypophysectomized at 28 days as well as the normal 60 day old rat both showed much more advanced development The main epiphyseal plate was fully resorbed and a separate ossification center appeared in the medial epicondyle (Figure 38 B and 38 C)

Rats hypophysectomized at intermediate ages (13 and 21 days) showed intermediate stages of development The skeletal ages attained by these rats at chronologic ages of 60 days are shown in Table XIX It will be noted that irrespective of the age at hypophysectomy the advance in skeletal age was constant being between 18 21 days

TABLE XIX

Estimated Skeletal Age in Days at 60 Days Chronological Age Following Hypophysectomy at Progressively Younger Ages

Age at Hypophysectomy	Skeletal Age at 60 days	Advance in Skeletal Age
28	47	19
21	42	21
13	33	20
6	24	18

Adapted from Asling *et al*[110]

The growth of animals hypophysectomized at varying ages appeared to cease at a constant age rather than after a constant interval Figures 39 and 40 illustrate the increase in body weight and in tail length and show that the increase in size persisted in all groups until about 30 days of age Thus rats hypophysectomized at early ages may make appreciable gains in weight and length as had been observed by earlier workers[111 112]

It would therefore appear that skeletal differentiation is under a control different from growth In seeking an explanation for the continued differ

[111]Collip J B Selye H and Thomson, D L Beitrage zur Kenntnis der Physiologie des Gehirnanhanges *Arch Path anat u Phys* 290 23-46 (1933)

[112]Van Eck, W F and Freud J Analysis of the Growth of Very Young Hypophysectomized Immature Rats *Acta Brev Neerl* 11 43-46 (1941)

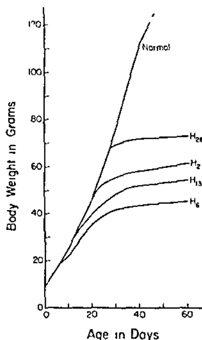


Fig 39 Curves Showing Gain in Body Weights of Rats Hypophysectomized at 6 13 21 or 28 Days of Age

After Walker *et al*[109]

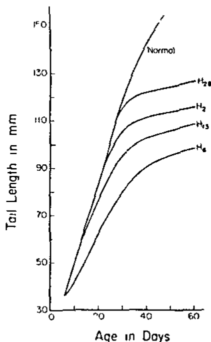


Fig 40 Curves Showing Increase in Length of the Tail of Rats Hypophysectomized at 6 13 21 or 28 Days of Age

After Walker *et al*[109]

entiation after hypophysectomy attention falls first on the thyroid hormone which is known to have effects for a number of days after administration [113] Thyroid hormonal effects manifested after hypophysectomy could be due either to continued low grade activity of the thyroid or to the persisting action of the thyroid hormone already present at the time of operation Persistence of action of pituitary hormones after hypophysectomy is very unlikely since their effects survive at most only a few hours [114]

THYROIDECTOMY

In evaluating the role of the thyroid gland the effects of thyroidectomy may first be examined Salmon[115, 116] had been able to thyroidectomize rats on the first day of life and Scow[100, 119] and Ray[101] in this laboratory also performed this operation In such rats growth was markedly retarded but *not completely arrested The skeletal age advanced to 18 days by a chrono*logical age of 60 days an advance of the same order as after hypophysectomy In rats which survived for 120 to 140 days the bone age advanced to 20 or 24 days This was in contrast to the arrest of differentiation observed in hypophysectomized rats after a three week advance Histologically the epiphyseal cartilage plates did *not* become sealed off from the marrow Some chondrogenesis continued and erosion of cartilage was also detectable The plates did not reduce in width with increasing chronological age as in normal rats, and thus at 56 days of age were actually wider than normal [120, 121] It is known that in the pituitary derangement which follows

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[113] Harrington C R *The Thyroid Gland its Chemistry and Physiology* H Milford Oxford University Press London (1933)

[114] Van Dyke D C Simpson M E Li C H and Evans H M Survival in the Circulation of the Growth and Adrenocorticotropic Hormones as Evidenced by Parabiosis *Am J Physiol* **163** 297 309 (1950)

[115] Salmon T N Effect of Thyro parathyroidectomy in Newborn Rats *Proc Soc Exp Biol and Med* **35** 489 491 (1936)

[116] Salmon T N The Effect on the Growth Rate of Thyro parathyroidectomy in Newborn Rats and of Subsequent Administration of Thyroid Parathyroid and Anterior Hypophysis *Endocrinol* **23** 446 457 (1938)

1 [illegible] velopment of Rats

1 [illegible] t of Thyro para [illegible] elopment of Rats
J Dent Res **19** 93 102 (1940)

[119] Scow R O and Simpson M E Thyroidectomy in the Newborn Rat *Anat Rec* **91** 209 226 (1945)

[120] Becks H Simpson M E Scow R O Asling C W, and Evans H M Skeletal Changes in Rats Thyroidectomized on the Day of Birth and the Effects of Growth Hormone in Such Animals Tibia Metacarpal and Caudal Vertebrae *Anat Rec* **100** 561 576 (1948)

[121] Becks H Scow R O Simpson M E Asling C W Li C H and Evans H M Response by the Rat Thyro parathyroidectomized at Birth to Growth Hormone and to Thyroxin Given Separately or in Combination III Skeletal Changes Tibia Metacarpal and Caudal Vertebrae, *Anat Rec* **107** 299-318 (1950)

thyroidectomy the alpha cells (the presumed source of growth hormone) are still recognizable though only in small numbers [122] It might be assumed that the secretion of growth hormone in small amounts continues thus accounting for the slow but sustained growth However as will be shown it is unlikely that this would explain the skeletal maturation which may still be slowly progressive two to four months after the operation The most complete retardation of both growth and maturation should be induced by combining removal of the thyroid and the pituitary gland eliminating sources of both thyroid and growth hormone

Combined Thyroidectomy and Hypophysectomy

In collaboration with R D Ray and D G Walker thyroidectomy on day 1 has been combined with hypophysectomy on day 20 Growth was markedly retarded in these rats Their skeletal age was the same as that after thyroidectomy alone being 18 days at 60 days chronological age Histologically the bones resembled more closely those of thyroidectomized rats *in that sealing off of the cartilage plates by bones did not develop* The retardation of growth and maturation was the most complete which had been attained

Effects of Hormone Administration on Skeletal Development

Growth Hormone Therapy

The growth hormone is unique among the pituitary hormones in that it is effective in the absence of the known target organs It has been shown to induce growth in hypophysectomized adrenalectomized rats [123] hypophysectomized adrenalectomized thyroidectomized rats (unpublished work) hypophysectomized thymectomized rats [124] and gonadectomized rats [125] The analysis here will be confined to studies of its action in normal rats and in

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[122] Koneff A A Scow R O Simpson M E Li C H and Evans H M Response by the Rat Thyro parathyroidectomized at Birth to Growth Hormone and to Thyroxin Given Separately or in Combination II Histological Changes in the Pituitary *Anat Rec* 104 465 476 (1949)

[123] Simpson M E Marx W Becks H and Evans H M Response of Adrenalectomized hypophysectomized Rats to the Pituitary Growth Hormone *Endocrin* 35 234 240 (1944)

[124] Reinhardt W L Marx W and Evans H M Effect of Pituitary Growth Hormone on the Thymectomized Rat *Proc Soc Exper Biol and Med* 46 411-415 (1941)

[125] Evans H M and Simpson M E Hormones of the Anterior Hypophysis *Am J Physiol* 98 511 546 (1931)

hypophysectomized, thyroidectomized, and thyroidectomized hypophysectomized animals

Growth Hormone in Normal Rats

If growth hormone therapy is instituted after the growth plateau has been attained in adulthood (starting at six to seven months of age) growth in body weight and length is resumed, and gigantism eventually results It will be remembered that only epiphyseal plates belonging to the delayed fusing group still remain to be reactivated The bones possessing these plates participate in the overgrowth of the animals Even after 14 months of treatment, when bone lengths 15% in excess of normal have been attained, the epiphyses remain open and responsive Other bones, notably those of the paws where fusion has already occurred at the single epiphyseal center, are unresponsive and thus the paws of these giant rats remain of only normal size [126]

If growth hormone administration is started in younger rats, marked differences are disclosed in the responsiveness of different parts of the skeleton The responsiveness is apparently related to the time of fusion characterizing individual bones Generally, all epiphyses will respond to growth hormone unless they are too near their time of normal fusion when treatment is begun Growth hormone administration starting at 81 days, 20 days before the time of fusion of the metacarpal epiphysis, did not cause elongation of the bone Its epiphyseal plate disappeared at the normal time [127] The tibias of the same animals grew substantially In the thirty-day injection period they attained their definitive length not usually reached until three or four months later [128] However, all of this growth could be attributed to activation of the proximal epiphyseal plate, for epiphyseal fusion took place at the normal time at the distal end of this bone (90 100 days of age)

In normal rats, therefore, growth hormone neither delays nor accelerates epiphyseal fusion The response of the various bones differs with the differences in their normal characteristics of growth and maturation

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[126] Evans H M, Becks, H, Asling C. W, Simpson M E, and Li C H The Gigantism Produced in Normal Rats by Injection of the Pituitary Growth Hormone IV Skeletal Changes Tibia Costochondral Junction and Caudal Vertebrae *Growth* 12 43 54 (1948)

[127] Asling C W Becks, H Simpson M E, Li C H, and Evans H M The Effect of Anterior Hypophyseal Growth Hormone on Epiphyseal Closure in the Third Metacarpal of Normal Female Rats *Anat Rec* 101 23 32 (1948)

[128] Asling, C W, Simpson, M E, Li, C H and Evans, H M Differences in the Response to Growth Hormone of the Rat's Proximal and Distal Tibial Epiphyses, *Anat Rec* 107 399 408 (1950)

Growth Hormone in Hypophysectomized Rats

Prompt increase in weight and skeletal dimensions occurs when growth hormone is injected into hypophysectomized rats All epiphyseal plates present respond to chondrogenesis cartilage erosion and elaboration of new delicate bone trabeculae Hypophysectomized rats are more sensitive to growth hormone than normal rats and so less hormone is required to produce a given increment If administered immediately after hypophysectomy endochondral ossification and growth of the bone is maintained Post operative intervals of over a year may elapse before instituting growth hormone treatment and yet the resumption of growth may become evident in five days [129]

The increase in width of the proximal tibial epiphyseal plate is within certain limits of dose and time proportional to the dose of hormone administered [130] This has allowed the development of a reliable method of assay of the hormone[131, 132] in which total doses of as little as five micrograms spread over a four day period may be detected Although small increases in the width of the cartilage below the unit defined for growth hormone can be produced by other hormones none evokes continued growth or growth proportional to dose [133]

No question can arise as to whether or not growth hormone delays epiphyseal fusion in hypophysectomized rats for the operation itself has already resulted in indefinite postponement of fusion Whether or not it hastens fusion must be considered with the problem of whether fusion occurs when the definitive length of the bone has been reached or at a characteristic chronological age or after the elapse of a definite period of activity Silberberg and Silberberg have maintained that fusion occurs when as a result of exogenous or endogenous stimuli the bones have

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[129] Becks H Simpson M E Evans H M Ray R D Li C H and Asling C W Response to Pituitary Growth Hormone and Thyroxin of the Tibias of Hypophysectomized Rats After Long Postoperative Intervals *Anat Rec* 94 631-656 (1946)

[130] Becks H Kibrick E A Marx W and Evans H M The Effect of Different Dose Levels of Growth Hormone on the Tibia of Young Hypophysectomized Female Rats *Growth* 4 437 447 (1941)

[131] Evans, H M Simpson M E Marx W and Kibrick E A Bioassay of the Pituitary Growth Hormone Width of the Proximal Epiphyseal Cartilage of the Tibia in Hypophysectomized Rats *Endocrinol* 32 13 16 (1943)

[132] Greenspan, F S Li C H Simpson M E and Evans H M Bioassay of Hypophyseal Growth Hormone The Tibia Test *Endocrinol* 45 455-463 (1949)

[133] Marx W Simpson, M E and Evans H M Specificity of the Epiphyseal Cartilage Test for the Pituitary Growth Hormone *Proc Soc Exper Biol and Med* 55 250-252 (1944)

reached full size[134] It appears, however, that growth hormone does not hasten fusion but that fusion may occur at certain centers during the chronic administration of growth hormone Analysis of the response of the metacarpal in hypophysectomized rats injected chronically with growth hormone is instructive[135] Epiphyseal fusion had not occurred after 8 months of treatment although the definitive length had been attained This was 6 months after the normal time of fusion The epiphysis was found fused at autopsy however after 14 months of injections It had not contributed further to the length of the bone during this time Other epiphyses of those normally fusing by 120 days of age were also found to be fused at the end of the experiment Those epiphyses which normally remain patent until senescence were open and active at the end of the experiment Thus the bones having such epiphyses attained greater than normal lengths Comparison of the skeletal proportions of these rats with intact rats treated with growth hormone disclosed that the incidence of disproportions was greater in the hypophysectomized group[109] They occurred notably in the paws[136] skull and pelvis

Figure 41 shows the length of the tibia in rat giants intact and hypophysectomized in which the experimental conditions were held closely comparable except for the hypophysectomy which took place when the rats had reached their growth plateau The doses of growth hormone were similar in the two groups The tibias of the hypophysectomized giants were much longer than those of the intact giants It is tempting to assume that this excess resulted from the absence of other pituitary factors which would have retarded growth The final explanation, however, may not be so simple for in early experiments on inducing gigantism in rats,[125] using preparations undoubtedly containing appreciable amounts of other pituitary factors, the resulting body dimensions were some of the largest which have ever been obtained

Growth Hormone in Thyroidectomized Rats

Rats thyroidectomized on the first day of life increased both in weight

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[134] Silberberg M and Silberberg R Influence of the Endocrine Glands on Growth and Aging of the Skeleton *Arch Path* 36 512 534 (1943)

[135] Becks H Asling C W Simpson M E Li C H, and Evans M H The Growth of Hypophysectomized Female Rats Following Chronic Treatment with Pure Pituitary Growth Hormone III Skeletal Changes Tibia Metacarpal *Costochondral* Junction and Caudal Vertebrae *Growth* 13 175 189 (1949)

[illegible] njected rats are difficult to reconcile with the hough these paws did not grow beyond normal were markedly thickened so that the digits

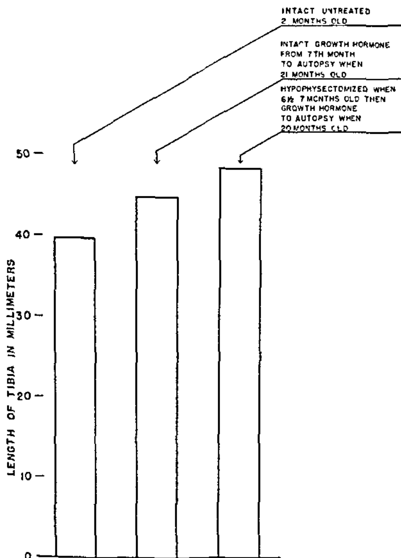


Fig 41 Graph Showing Length of Tibia of Intact and of Hypophysec tomized Rats Injected Chronically with Growth Hormone

and length when injected with growth hormone [100, 101, 137] Bones throughout the body were stimulated [120, 121] The animals were relatively insensitive to the hormone and required 25 times as much as hypophysectomized rats to induce equivalent growth *Skeletal maturation was not enhanced by* administration of the hormone

Growth Hormone in Thyroidectomized Hypophysectomized Rats

Rats deprived of both thyroid and pituitary glands responded to growth hormone with growth They were *relatively insensitive to the hormone No* skeletal maturation occurred and the animals showed the same bone age as the untreated controls 18 days at 60 days chronological age

THYROXIN THERAPY

Thyroxin in Normal Rats

Thyroxin caused no increase in body length in normal rats [138] *Smith and* McLean produced premature fusion of the proximal tibial epiphysis[139] with thyroid treatment H V Christensen and I M Carlson produced premature epiphyseal closure in the metacarpal of intact rats in this laboratory by thyroid feeding (unpublished) Noback *et al* [140] reported acceleration of development of epiphyseal centers in newborn rats under thyroxin therapy

Thyroxin in Hypophysectomized Rats

The capacity of thyroxin to induce growth of hypophysectomized rats *is still under evaluation At marginally* toxic levels *(over 5 µg) no growth* was obtained [123] Barely detectable growth occurred at 5 µg [107] At 3 to 3 µg dosages small but statistically significant weight and length increases *were demonstrated (Ray et al* [104] *also* C P *Williams unpublished)* The question arose as to whether this growth might be sustained In an experiment now in progress weight increase continued for some months at a 2 µg dosage *Body length also increased as shown in Figure 42* The

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137 Scow R O and Marx W *Response to Pituitary Growth Hormone of Rats* Thyroidectomized on the Day of Birth *Anat Rec* **91** 227 236 (1945)

138 Evans H M Simpson M E and Pencharz R I Relation Between the Growth Promoting Effects of the Pituitary and the Thyroid Hormone *Endocrinol* 25 175 182 (1939)

139 Smith E E and McLean F C of Hyperthyroidism Upon Growth and Chemical Composition of Bone *Endor* 552 (1938)

140 Noback C R Barnett J C a H S The Time of Appearance of Ossification Centers in the Rat as I tions of Thyroxin Thiouracil Estradiol and Propionate 49-67 (1949)

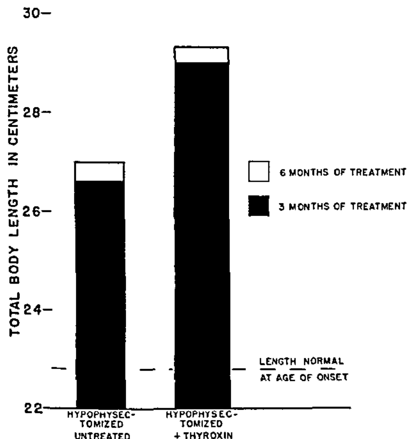


Fig 42 Graph Showing Total Length of Rats Injected Chronically with Thyroxin Starting Immediately after Hypophysectomy at 28 Days of Age

The dosage of thyroxin was 2.0 μg daily. The lengths were measured under anesthesia at 3 months and again at 6 months

magnitude of these increases of course in no respect approached that of normal rats or of hypophysectomized growth hormone treated rats

Skeletal differentiation continues in thyroxin treated hypophysectomized rats demonstrating that the maturing effect of this hormone is not mediated by the pituitary gland[99 107 141] Table XX shows the advance may be maintained at a virtually normal rate During chronic injection mentioned above roentgenograms of hypophysectomized rats after three months of treatment showed that all except the late fusing epiphyses (such as that of the proximal end of the tibia) had fused Even after 6 months of treatment however patency was maintained in the late fusing group

In hypophysectomized rats the fusion of epiphyses was effected by continued erosion of the cartilage plate in the absence of chondrogenesis Marrow contact with the cartilage was maintained and sealing off by bone did not occur At the chondro medullary junction chondroclasts were found but capillary tufts were sparse[104] Long after hypophysectomy when the sealing bone had been present for long periods the administration of thyroxin resulted in breaking up of this bony lamina and re establishment of marrow contact with the cartilage[1 9]

Thyroxin in Thyroidectomized Rats

A part of the reaction of thyroidectomized rats to thyroxin is undoubt

TABLE XX

Skeletal Age of Female Rats Hypophysectomized at 21 Days of Age and Injected with Thyroxin between 30 to 60 Days of Age

Treatment	Skeletal Age		Advance in Skeletal Age
	At 30 Days	At 60 Days	
Hypophysectomized Untreated	29	42	13
Hypophysectomized + Thyroxin (2 5 μg per day)	29	55	26
Normal Untreated	30	60	30

After Ray *et al*[104]

edly due to the repair of the pituitary gland and resumption of its secretion [12] With the resultant increase in endogenous growth hormone the question becomes one of the reaction to thyroxin and growth hormone together The discussion will therefore be deferred to that section It is also convenient to defer discussion of thyroxin in thyroidectomized hypophysectomized rats

Combined Thyroxin and Growth Hormone Therapy

Thyroxin with Growth Hormone in Normal Rats

It has been known for some time that thyroxin augments the growth resulting from growth hormone injections in normal rats [138] but the skeletal maturation has not been studied

Thyroxin with Growth Hormone in Hypophysectomized Rats

When these two hormones were injected into hypophysectomized rats both growth and maturation occurred [89, 139, 141] A delicate balance of dosage is required otherwise one or the other effect may predominate [141] The proximal epiphyseal plate of the tibia may show greater widths than with growth hormone alone under one experimental regimen [89] or may be much *narrower than with the growth hormone alone (C P Williams unpublished)* However, the bones always attain greater dimensions than with growth hormone alone although epiphyseal closure may then supervene before normality of length is attained It has been reported that at one site the mandibular condyle thyroxin inhibited the response to growth hormone [142] This inhibition was judged by the width of the condylar cartilage In another experiment however when the condylar segment of the mandible was measured, it was found that the response to growth hormone was augmented as in other bones (Table XXI) The histological picture corresponded closely to that observed in the former experiment

Thyroxin and Thyroxin with Growth Hormone in Thyroidectomized Rats

The resumption of growth when thyroxin is administered to thyroidectomized rats was as striking as that resulting from the administration of growth hormone to hypophysectomized rats The growth rate was equal to that of normal rats Skeletal differentiation was also accelerated leading to a rate of maturation even in excess of normal In rats thyroidectomized

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[14] Collins D A Becks H Simpson M E and Evans H M Growth and Transformation of the Mandibular Joint in the Rat *Am J Orth Ow and Oral Surg* 32 431-451 (1946)

TABLE XXI

Length of Condylar Segment of Mandible of Female Rats Hypophysectomized at 28 days of Age and Injected for the Ensuing 40 Days with Growth Hormone and/or Thyroxin *

Group	Number of Rats	Distance Between Crest of Condyle and Posterior Alveolar Margin of Last Molar Tooth
Untreated Controls	5	8 4+ 0 22 mm
Growth Hormone (50 200 μg **)	4	9 6± 0 09 mm
Thyroxin (2 5 μg)	5	8 7± 0 12 mm
Growth Hormone and Thyroxin (doses as specified above)	5	10 3± 0 22 mm

*Unpublished work with C P Williams
**Dosage increased with increasing body weight

on the first day of life daily doses of thyroxin of 2 5 to 3 0 μg so stimulated differentiation that in thirty days of treatment the bone age advanced 35 40 days even though the treatment had not started until 30 days of age when the bone age was 15 to 18 days Scow *et al*[100] felt that when the two hormones were administered concurrently no advantage in growth was seen over that when thyroxin was administered alone since maximum growth already was occurring under the stimulus of the endogenous growth hormone evoked by thyroxin therapy Ray *et al*[101] using slightly higher doses of growth hormone reported augmentation of the growth by the concurrent administration of the two hormones so that the rate exceeded that of normal rats In both instances the bone age advanced at the same rate as with thyroxin alone

Thyroxin and Thyroxin with Growth Hormone in Thyroidectomized hypophysectomized Rats

When thyroidectomy on day 1 was followed by hypophysectomy on day 20 thyroxin administered from day 30 to day 60 caused slight but significant increases in body weight body length and the lengths of individual bones

Such doubly deficient animals could not of course respond to thyroxin by production of endogenous growth hormone as could thyroidectomized rats Although the growth elicited by growth hormone injections was only slightly greater than that with thyroxin the combined administration of these two hormones resulted in restoration of a normal rate of growth Figure 43 illustrates the relative effectiveness of thyroxin and growth hormone alone and combined in stimulating body weight in hypophysectomized thyroidectomized and thyroidectomized hypophysectomized rats In body length and bone lengths these animals responded in a manner corresponding to their body weights

Skeletal differentiation was resumed at normal or greater than normal rates when thyroxin was administered, the addition of growth hormone did not further advance this rate In fact in the doubly injected rats the stimulus to cartilage erosion was balanced by chrondrogenesis, and a tendency to premature epiphyseal union (as in the medial humeral epicondyle) was corrected This is illustrated in Figure 44 showing the distal end of the humerus in doubly deficient rats injected with the two hormones alone or in combination

In recapitulation, it appears that in rats growth hormone is a potent stimulus to skeletal growth but has little effect on maturation while thyroxin directly advances maturation but stimulates growth only slightly By administering these hormones concurrently in properly balanced dosages a normal balance between growth and differentiation may be established The necessity of both hormones for this balance is best shown by studying their actions in thyroidectomized hypophysectomized rats

Adrenocorticotropic Hormone in Normal Rats

In normal rats ACTH injections (1 mg daily) retarded body growth[143, 144] and chondrogenesis and osteogenesis at the proximal tibial epiphysis[145, 146] though the retardation was not found to be as severe as after hypophysec

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[143] Evans H M, Simpson M E and Li C H Inhibiting Effect of Adrenocorticotropic Hormone on the Growth of Male Rats *Endocrinol* 33 237 238 (1943)

[144] Baker, B L Ingle D J Li C H and Evans H M The Effect on Liver Structure of Treatment with Adrenocorticotropin Under Varied Dietary Conditions *Am J Anat* 82 75 103 (1948)

[145] Becks, H Simpson, M E Li C H and Evans H M Effects of Adrenocorticotropic Hormone (ACTH) on the Osseous System in Normal Rats *Endocrinol* 34 305 310 (1944)

[146] Baker B L and Ingle D J Growth Inhibition in Bone and Bone Marrow Following Treatment with Adrenocorticotropin (ACTH) *Endocrinol* 43 442-420 (1948)

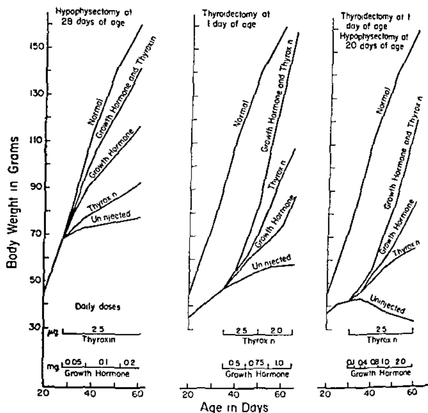


Fig 43 Curves Showing Body Weight Response of Hypophysectomized, Thyroidectomized and Thyroidectomized-hypophysectomized Rats to Growth Hormone, Thyroxin, and the Combined Treatment

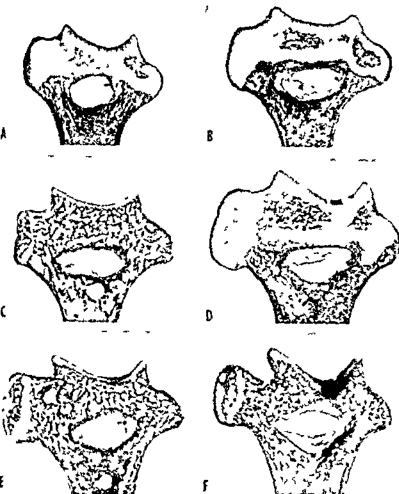


Fig. 44. Response of Distal Humeral Epiphysis of Thyroidectomized-hypophysectomized Rats to Thyroxin, Growth Hormone and the Combination.

The animals were 60 days of age except as noted. (H & E stain. × 10.) *A*. Normal rat 18 days of age, bone age control. The main epiphyseal ossification center is present. *B*. Thyroidectomized day 1, subsequently hypophysectomized day 30. Only the main epiphyseal ossification center is present. *C*. Both operations described in *B*, treated with thyroxin 25 µg daily day 30 to 60. The main epiphyseal cartilage plate and the plate in the medial epicondyle are both perforated. *D*. Both operations described in *B*. Growth hormone dose increasing from 0.1 to 2.0 mg daily day 30 to 60. Increase in size of the bone but no further advance in ossification beyond the doubly operated control. *E*. Both operations described in *B*. Growth hormone and thyroxin (both as above). The main epiphyseal cartilage plate has disappeared. That in the medial epicondyle is intact and active. *F*. Normal rat 60 days of age, chronological age control. The main epiphyseal cartilage plate has disappeared. The plate in the medial epicondyle is still active.

tomy The growth inhibition and bone changes were not caused by ACTH in the absence of the suprarenals Therefore, in view of the effectiveness of the hormone through the suprarenal cortex, the cortical steroids would be expected to have a similar antagonistic action [147, 148]

Adrenocorticotropic Hormone in Hypophysectomized Rats

In hypophysectomized rats ACTH did not affect the already inactive condition of tibial epiphysis However, when ACTH was administered concurrently with growth hormone, marked inhibition of the action of growth hormone was demonstrated [149, 150] In none of these experiments had skeletal maturation been studied (Such studies are especially desirable in view of numerous reports of precocious skeletal maturation in human beings suffering from hyperplasia of the suprarenal cortex)

Selection of rats hypophysectomized at an age earlier than that in the preceding experiments (26 28 days) offered a specific advantage With earlier hypophysectomy retardation of skeletal age would be more obvious, and any effect of the hormone on this phase of morphogenesis would accordingly be easier to detect Therefore, ACTH therapy was initiated the day following hypophysectomy at 21 days of age, and simultaneously in normal rats of the same age, males being used Figure 45 is a photograph showing such rats at the end of the experiment, when 60 days of age Figure 46 is a graph showing average body weight of the various groups, injected and control The suppression of growth in the injected rats is obvious Table XXII gives the body weight and length, and measurements of some of the long bones, also illustrating the growth inhibition

Histologically, marked narrowing of the proximal *tibial epiphyseal* plate was found The intact controls averaged 330 micra the intact treated rats 207 micra, the hypophysectomized controls 291 micra, and the hypophysec

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[147] Ingle, D J , Higgins, S M , and Kendall, E C Atrophy of the Adrenal Cortex *in the* Rat Produced by Administration of Large Amounts of Cortin, *Anat Rec* 71 363 372 (1938)

[148] Winter, C A , Silber R H , and Stoerck, H C Production of Reversible Hyperadrenocortinism *in Rats by Prolonged Administration* of Cortisone *Endocrinol* 47 60-72 (1950)

[149] Marx, W , Simpson M E ; Li C. H , and Evans, H M Antagonism of Pituitary Adrenocorticotropic Hormone to Growth Hormone in Hypophysectomized Rats *Endocrinol* 33 102-105 (1943)

[150] Becks, H , Simpson, M E , Marx, W , Li C H , and Evans H M Antagonism of Pituitary Adrenocorticotropic Hormone (ACTH) to the Action of Growth Hormone on the Osseous System of Hypophysectomized Rats *Endocrinol* 34 311 316 (1944)

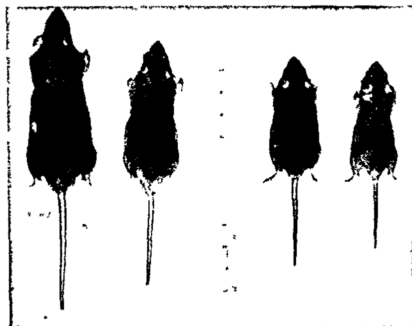


Fig 45 Male Rats 60 Days of Age after 39 Days of Treatment with Adrenocorticotropic Hormone (ACTH)

One third natural size *From left to right* intact untreated intact ACTH (2 0 mg daily from day 22 to day 43 then 3 0 mg daily until day 60) hypophysectomized at day 21 untreated hypophysectomized at day 21 ACTH (2 0 mg daily from day 22 to day 60)

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[148] Winter C A Silber R H and Stoerck H C Production of Reversible Hyperadrenocorticism in Rats by Prolonged Administration of Cortisone *Endocrinol* 47 60-72 (1950)

[149] Marx W Simpson M E Li C H and Evans H M Antagonism of Pituitary Adrenocorticotropic Hormone to Growth Hormone in Hypophysectomized Rats *Endocrinol* **33** 102 105 (1943)

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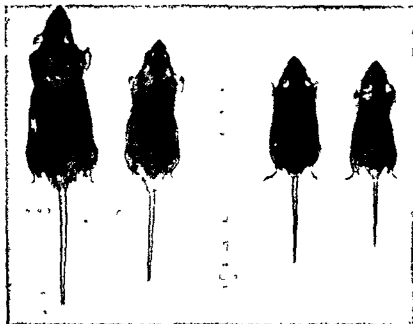


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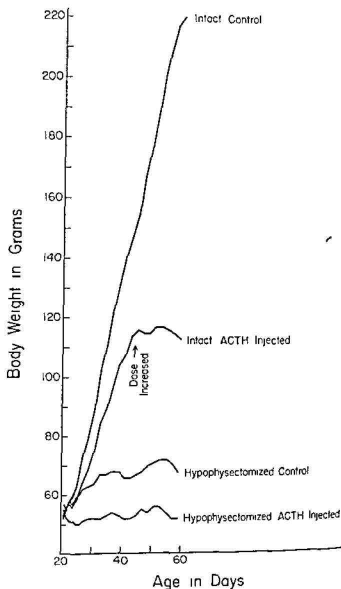


Fig 46 Curve Showing Body Weight Response of Rats Treated with ACTH, as Specified in Figure 45

TABLE XXII

Measurements and Skeletal Age of Male Rats, Intact and Hypophysectomized, Untreated and Treated with ACTH from 22 to 60 Days of Age *

Group	Number of Rats	Bone Lengths at Autopsy			Total Body Length		Normal Age Equivalent of Body Length *(days)*	Skeletal Age at Autopsy *(days)*
		Tibia *(mm)*	Femur *(mm)*	Humerus *(mm)*	At Autopsy *(cm)*	Gain During Injection Period *(cm)*		
Intact Untreated	7	35.6	31.1	24.7	36.9	16.3	60	60
Intact + ACTH	5	30.3	26.8	21.7	29.8	10.0	38	60
Hypophysectomized Untreated	4	26.7	22.4	19.1	24.8	4.4	29	>35 <40
Hypophysectomized + ACTH	4	24.4	20.5	18.0	22.1	1.6	25	>35 <40

*Hypophysectomy at 21 days of age. ACTH dosage for intact rats 2.0 mg/day *during first half of experiment*, 3.0 mg/day thereafter; dosage for hypophysectomized rats 2.0 mg/day throughout

tomized treated 163 micra The epiphyseal plates in the treated groups showed irregularity of chondrocyte columns and diminished cellularity Though excessive bony resorption had been anticipated quite the opposite was found *The bony trabeculae extended far considerable distances into* the marrow cavity both in intact and hypophysectomized treated rats and showed lack of resorption and re organization They thus contrasted on the one hand with the normal controls which showed active bony reorganization and on the other with the hypophysectomized controls which showed resorption of the spongiosa In treated rats the cartilage cores were invested by bone up to the chondro-medullary junction as opposed to the normal delicacy of the trabeculae in this region The surface of these trabeculae were intensely basophilic with haematoxylin The costochondral junctions showed the same response as the tibias and are illustrated in Figure 47

The skeletal age was neither accelerated nor retarded by the treatment In Table XXII the skeletal age and the length age (i e age of normal rats of equivalent length) are presented The degree of differentiation *attained by the distal end of the humerus added support to the skeletal age* diagnoses (Figure 48) The intact treated rats showed normal differentiation the hypophysectomized treated rats were retarded as in the hypophysectomized controls

Although food consumption was not measured in this experiment the evidence from experiments cited earlier[11] was that food consumption was not a factor in the suppression of growth with ACTH since rats treated with ACTH ate as much as if not more than rats growing actively *in* response to growth hormone and when the two hormones were administered concurrently the food intake increased further Furthermore marked restriction of the food intake of normal rats inhibited growth less than did ACTH administration[115]

Effects of Protein Deficiency on Skeletal Development

H I Dougherty and M M Nelson have recently succeeded in main*taining a group* of male rats until autopsy at 60 days of age on a diet containing no protein having instituted the regimen at weaning (21 days of age) The experimental *period was thus identical* with that in the experiment just described Although they are not strictly comparable with the hypophysectomized ACTH treated rats it is interesting to examine the effects on skeletal morphogenesis of these two forms of severe protein metabolism disturbance Table XXIII shows their weight *length (total* body and tibia) and skeletal age Their weight loss was greater than that of ACTH treated hypophysectomized rats their length gain slightly less

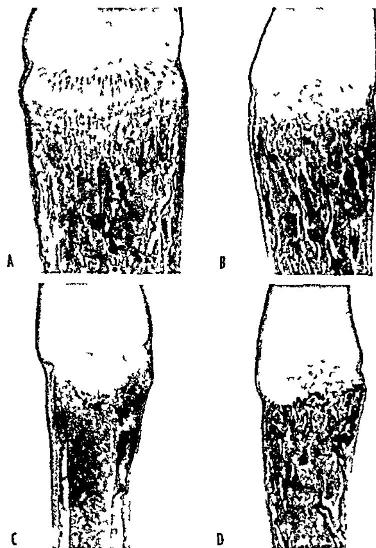


Fig 47 Costochondral Junction of Rats Treated w th ACTH as Speci fied in Figure 45

(H & E sta n × 40) *A* Intact untre
n m l

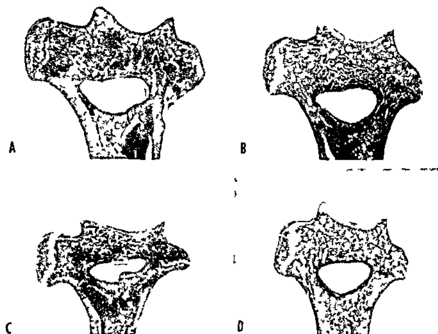


Fig 48 Response of Distal Humeral Epiphysis of Intact and Hypophysectomized Rats to Treatment with ACTH as Specified in Figure 45

(H & E stain × 10) *A* Intact untreated Closure of the main epiphyseal plate has occurred while that in the medial epicondyle remains patent *B* Intact ACTH

TABLE XXIII

Measurements of Protein Deficient Rats and of Hypophysectomized ACTH Treated Male Rats *

Measurement	Experimental Regimen		
		Hypophysectomy	
	Protein Deficiency **	+ACTH 2 0 µg /day	No Treatment
Body Weight (gm)			
At Autopsy	34 0	51 5	68 3
Gain	—16 2	—2 0	11 0
Body Length (cm)			
At Autopsy	21 3	22 1	24 8
Gain	1 0	1 6	4 4
Tibia Length (mm)	24 5	24 4	26 7
Skeletal Age (days)	>40 <50	>35 <40	>35 <40

* All rats 21 days of age at onset 60 days of age at autopsy

** Purified diet containing no protein experiment in collaboration with H L Dougherty and M M Nelson

their tibia length the same, and their skeletal age slightly more It will be noted however that some retardation of skeletal age did occur under these conditions Histologically the tibial epiphyseal plates were very narrow and even in some animals showed perforations, chondrocytes were very scanty The medullary cavity was devoid of bone except for the transverse lamina which sealed the cartilage plate from the aplastic marrow (Figure 49A) The distal end of the humerus showed some retardation of maturation (Figure 49B) The most marked contrast between the protein deficient dwarfs and the ACTH treated hypophysectomized dwarfs was in the marked osseous resorption in the former and the failure of osseous resorption in the latter

Conference Discussion

Armstrong Dr Asling we thank you for a very careful summary of a thorough study which undoubtedly was meticulously carried out over a long time a study in which much insight and imagination had to be brought to play

I do not know whether there are questions or further discus

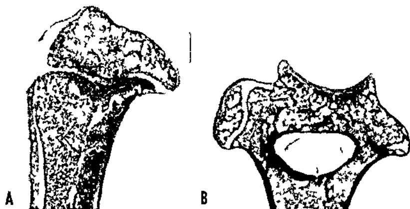


Fig 49 Representative Epiphyses of Rats 60 Days Old after Maintenance on a Diet Devoid of Protein since Weaning at 21 Days of Age

Experiment conducted in collaboration with H L Dougherty and M M Nelson (H & E stain, × 10) *A* Proximal tibial epiphysis The cartilage plate is narrow The spongiosa has been completely resorbed *B* Distal humeral epiphysis Persistence of a fragment of the main epiphyseal plate indicates slight retardation in differentiation

Asling I hope that the report has not been so long that we have lost the chance of hearing comment

Armstrong Are you always referring to females and males here?

Asling I refer only to females until the very last of the report Then I specify male

In general I can say that we have not noted any sex difference in the animal responses but I shall speak of that specifically The males do have a slightly higher body weight gain I think that their growth curve may have a more definitely upward inclination so that they may gain a cm or 1 5 cm over the course of a year and a half after having reached adult life They do not have a plateau as one might suspect

Bartter How do you calculate that advance?

Asling If they were 28 days of age at hypophysectomy and obtained ultimately a bone age of 47 days 28 from 47 was 19 When they stopped is very difficult to say At the time they are sacrificed histologic studies showed that this sealing had taken place very well As a matter of fact hypophysectomized animals even 2 years after hypophysectomy do not ever appear to get to a 60 day bone age They seem to hold to around 50 The rats hypophysectomized when 28 days old and sacrificed when 60 obviously have 32 days in which to make their advance in bone age The rats hypophysectomized at 6 days of age had 54 days in which to make it The advance however was the same amount

Armstrong I notice the upper one is missing some teeth Is that a common observation?

Asling The incisors are very poorly indicated in the photograph but are actually present Eruption of the teeth did go ahead and they appeared on time Teeth appear very early and apparently hypophysectomy even at this young age is not early enough to affect tooth maturation

Armstrong What about the third molar?

Asling I am sorry I cannot comment on that It doesn t show well on one side in the picture It was present in the specimen

Hodge How did you prepare those skulls?

Asling These were done on the bug table

Hodge Does that method make skulls in which you can see the suture lines?

Asling Some sutures such as those in the basicranium were inked to make them more visible in the photograph

Bezelander What is a bug table?

Asling A table of maggots that avidly consume connective tissue muscle and so forth but will leave the skeleton intact and very nicely cleaned

Follis These thyroidectomized animals still have their parathyroids?

Asling Let's see Scow[100] took out the parathyroid and found no difficulty whatever as long as he kept the mothers on a high calcium diet Ray[101] put the parathyroids back into the sternocleidomastoid muscle and found no advantage from this Neither of them got any tetany unless the mothers had a very poor diet This is a critical matter as to whether the parathyroid hormone of these animals has any effect Rats are rather resistant to parathyroidectomy anyway and this is a factor not yet studied in our experiments The parathyroid is gone in most of these animals

Follis Is that cortex thicker than one would expect normally?

Asling Yes and I wish I could understand more of the mechanism of the thickening The lines of apposition are found along both dorsal and ventral surfaces of the bone

I might add that periosteal osteogenesis also takes place under administration of growth hormone but the marrow cavity seems to expand in pace with it If one takes a cross section through the diaphysis of the tibia and measures the total area of it with a planimeter and then measures the marrow cavity the total area will increase with periosteal osteogenesis, but there will be endosteal absorption so that the proportion of the total area to marrow cavity remains the same The balance seems to be sustained

However it is not such an active process that one can find increased numbers of osteoblasts on the endosteal surface

Follis You apparently have not seen anything such as one would see in acromegaly with increased periosteal osteogenesis

Asling If I may translate the word 'acromegaly as 'enlarged prominences the best example I can give in these rats would be the marked increase along the temporal crest at the attachment of the temporal muscle Rats do not have any appreciable amount of sinuses so we do not see any sinus activation We see very marked activation of the mandibular condyle As to the occlusion between the molar teeth there may be an anterior displacement of the mandible The rat has a continuous working of the incisors one inside the other and they seem to keep on fitting together so that one does not get evidence of malocclusion of the incisors

The paws show the least correspondence to acromegaly The bones are no longer than normal but there is a thickening of the digits dorso-ventrally

and also laterally so that the animals hold their digits in a spread position

I should give one other example The deltoid tuberosities attain unusual prominence in these chronically injected animals

Certainly we are not prepared to say that acromegaly has been produced in rats One might be tempted to call some of these things acromegaly but I do not know that this is justified

Armstrong What is the number of micrograms of thyroxin?

Asling Micrograms of thyroxin—2 micrograms daily

Armstrong How is it administered?

Asling Subcutaneously 6 days a week giving them a rest on the seventh

The pituitary undergoes a considerable amount of degeneration in the thyroidectomized animal If one gives thyroxin one will find a marked repair of pituitary cytology in the animal It is difficult to differentiate these pictures from the normal animals For example gonadotropic action is also restored The animals go back into a relatively good state of health

Follis Is that milligram equivalent to commercial material?

Asling I do not know what Dr Li's conclusions are on the potency of his preparations as compared with commercial ACTH but I believe he finds his hormone more potent The hormone that I have had from Dr Li is very active according to adrenal maintenance tests and the ascorbic acid assay You will see the doses which have been necessary in order to demonstrate any skeletal response

I cannot say what these doses are equivalent to among the commercial materials

Follis Is that given in one injection or several?

Asling This was divided among three injections daily It might have been better to divide it among six a day

Armstrong Do you think you would have obtained the same type of result if you could have administered an adrenocortical extract which contained the cortical hormone?

Asling In *Endocrinology* of July 1950 there is a report on induced hypercortisonism[143] Winter and colleagues demonstrated inhibited body growth They did not use hypophysectomized animals they used normal animals The bone histology was mentioned briefly in this report skeletal growth was also inhibited by cortisone

Armstrong What you are doing then is adding one insult on top of another, is that a fair statement?

Asling ACTH does not have any effect on the skeleton in the absence of the adrenal cortex

Armstrong Exactly

Asling So that this whole response is through the adrenal cortex

Howard Did the animals eat as well as the controls?

Asling In this experiment the food intake was not measured The food intake has been measured by Dr Simpson on ACTH treatment with and without growth hormone ACTH treated animals ate more than the hypophysectomized controls and as much as if not more than animals growing rapidly as a result of growth hormone injections [149]

Pfeiffer What is the mortality of those animals?

Asling I lost 50 per cent of my animals in this experiment

Pfeiffer What from?

Asling Sudden death I think that the hormone had some posterior lobe principle in it

Bartter Were your ACTH treated animals obese?

Asling No they were emaciated

Pfeiffer One has about the same loss of mice with large doses of cortisone

Asling There is very high mortality in both groups Van Dyke in our laboratory succeeded in carrying much heavier rats at a 1 mgm per day level for 116 days with virtually no mortality These animals had already accomplished practically all of their growth and it would be very difficult to establish any substantial restriction of growth with them

Follis Why do you say you would expect restriction?

Asling I thought osteoporosis was one of the features of adrenal cortical hyperplasia Am I in error?

Sobel There have been reports to the effect that ACTH causes osteoporosis

Urist That may be due to its antianabolic effects It may depress the rate of bone formation and in that way produce osteoporosis

Follis Did you get any weight gain in the protein deficient animals?

Asling Minus 19 grams

Urist What is the mortality under such conditions?

Asling Forty per cent

Urist Did they get any amino acids?

Asling No, sir They had a sucrose fat and vitamin salt mixture and they had no protein whatever Forty per cent of them died before they got to 60 days of age At the time of autopsy some were becoming moribund, others looked as if they could have held on for another 5 or 10 days

McLean As long as the bone is not growing you cannot demonstrate failure to resorb

Asling The bone does resorb after hypophysectomy Estrogens do not prevent the resorption after hypophysectomy

McLean You mean the estrogen was given immediately after hypophysectomy?

Asling The estrogen was started on the day of operation and continued for 18 days

Pfeiffer How long after adrenalectomy was the estrogen treatment started?

Asling The estrogen treated animals were intact not adrenalectomized The ACTH level reached after approximately 50 hours of estrogen treatment corresponded to that reached 50 hours after adrenalectomy

Pfeiffer That is only a temporary response In a chronic estrogen treated animal the adrenal usually goes down I do not know what happens in a hypophysectomized animal I do not doubt that there is an initial ACTH increase

Asling You do not think this would be sustained?

Pfeiffer No

Sobel How did you measure this ACTH?

Asling Gemsell and Van Dyke did this by taking the plasma of the animals and making crude extracts to get an ACTH bearing fraction They went through part of the purification procedure of ACTH so that if ACTH was in the plasma they would have a powder which carried it Then they injected it into hypophysectomized rats and determined the amount of depletion of ascorbic acid in the adrenal cortex

Sobel The reason it is of interest is that we have some basis to believe that childhood convulsions of unknown origin have to do with sudden upsurges of ACTH I did not know there was a method for ACTH assay

Asling I believe this report will appear very shortly

Urist I would like to raise a question which may be answered by Dr Asling's very important experiments Does ACTH produce any specific or qualitative change in the structure of the growing bone?

Asling In the roentgenograms of the animals which I studied the density of the bone was greatest in the intact untreated rats In the next group it was not possible to differentiate between hypophysectomized untreated and intact treated both being moderately more radiolucent The radiographic density was least in the hypophysectomized ACTH treated animals Their bones were of course smaller and thinner They could pass the rays more easily in this same order

Histologically down in cortical bone well down into the diaphysis it was possible to see more of vascularity and even encroachment upon the cortical bone lessened density due to this vascularity and even some marrow elements

My only reservation is that I must cut cross sections of these bones to make sure that slight differences in orientation of the plane of section are not responsible for my impression that bone density was less in the ACTH treated animals than in their respective intact or hypophysectomized controls

Urist Have you observed anything that might be termed experimental osteoporosis?

Asling If I had a good definition of osteoporosis I should like to say that I got it experimentally but it still seems to be a roentgenographic diagnosis rather than a settled pathologic entity

I should like to hear your definition of osteoporosis Speaking pathologically osteoporosis in the cortical bone is certainly some form of rarefaction

Urist Using the word in the sense of porous bone having a thin cortex The pores being filled either with bone marrow or vascular spaces

Asling Subject to confirmation by cutting cross sections of the bones I would say the bone is more porous in the treated groups than in their controls The cortex is very much thinner in the treated group There was no evidence of increased numbers of osteoclasts

Follis I thought you made the point that there was more bone between the epiphyseal cartilage in the ACTH unhypophysectomized animals than there was in the normal

Asling The spongiosa underwent very poor if any resorption in the medullary cavity I am trying to differentiate carefully between the cortical

bone and the endochondromedullary bone here The two findings are in exact opposition, but perhaps they can be reconciled

Shorr Obviously, the thought in the back of the minds of the questioners is the relationship of your findings to certain pathologic states in man Am I right in thinking that your dosage of ACTH was selected with the idea that you must choose a dose that would produce some detectable change in the skeleton?

Asling Previous experiments of Becks Simpson, Li, and Evans[145] using doses of 1 mg, and occasionally more had not produced the maximal bone changes that I hoped to elicit

Shorr It would be helpful to learn from you the spread between the maintenance dose of ACTH in the preparation that you used and that which you found necessary to use in order to produce bone changes What was the spread?

Asling I believe that was 5 to 10 micrograms, which then is a factor of 500

Shorr That might give us some idea as to the relation of this action of ACTH to pathological states in man It was necessary to give about 500 times the maintenance dose of ACTH in order to produce what you would regard as definite changes in the bony skeleton?

Asling On reconsideration, I have given you the wrong answer The figure 5 to 10 micrograms and the factor of 500 derived from it represent the minimum dose which will give a detectable response in the adrenal repair test in hypophysectomized rats The maintenance dose is substantially higher, perhaps ranging between 0 5 to 1 0 mg, depending on the preparation This would mean that in the experiments I was using 3 to 6-fold the maintenance dose

Shorr Throughout the course of this chronic administration, did you look at the adrenals and did you find that they were maintaining a state of good storage of preformed lipids or were they exhausted? What was the state of the adrenals?

Asling The adrenals were, of course, hypertrophic They were enlarged I cannot give you the figure I believe that the intact animals had about 80 mg adrenals The fat storage indicated enormous stimulation

Shorr There was plenty of lipid right throughout?

Asling Full

And there were enormously thickened cortices

As I mentioned in the hypophysectomized animals what with the diminished body tone and the increased dimensions of the adrenal the gland was actually palpable

The ascorbic acid depletion test was run on the adrenals of the hypophysectomized animals It did not seem sound to do this on the intact animals because of the possibility of alarm reaction at the time of autopsy confusing the results In the untreated hypophysectomized animals the ascorbic acid level was already rather low 40 days after operation In the treated hypophysectomized animals further reduction in ascorbic acid was found

Shorr Initially or throughout?

Asling This was an ascorbic acid assay on the adrenal gland itself at autopsy after 39 days of treatment

The gonads of the intact animals were depressed beyond body weight proportion The balanopreputial cleavage which is an index of the process of sex maturation in the male (just as the vaginal opening would be in the female) was retarded The hypophysectomized animals showed little if any stimulation

I suspect that there was a trace of ICSH which did not appear with conventional assay techniques but which showed up under the high dosages and under the long term of administration

This might be true of all pure pituitary hormones Perhaps if you inject them at high enough dosage—fiftyfold of the M E D and for a long enough time—6 weeks you may be able to disclose contamination with traces of hormone other than the primary one

Reifenstein Dr Asling here is a more general question that has worried me a little bit in connection with the problems posed by your presentation I wonder what your feeling is about the distortion that might come into your results from the fact that you are replacing in the hypophysectomized animal only one tropic substance whereas the animal is deficient in all tropic substances?

Asling It would be most instructive I think to run a single deficiency experiment instead of a single repair I should like to give five pure hormones and leave out the sixth

Reifenstein Do you think that this fact is going to change your results or don t you have any guesses on this point?

Asling I had better not hazard them I think the interaction of these hormones is distinct sometimes remarkable The one which we feel certain is a physiologic relationship is the growth hormone—thyrotropic hormone

synergism I do not believe we need to have reservations awaiting the final purification of thyrotropic hormone to say that growth hormone and thyrotropic hormone will be found to be synergistic What in addition we shall gain by inclusion of the gonadotropins I do not know I wish somebody would tell me why sex hormones are not effective in skeletal maturation in these experiments when they are so conspicuous a part of human skeletal maturation for example I am most eager to find out what will happen there

Pfeiffer You will have to examine imbalances between the genoadotrophins and other pituitary hormones and sex hormones I think in order to get an answer to that I mean I think it is a balance situation

Asling I have wondered whether some of the effects of the sex hormone are mediated through the pituitary even in pituitary deficient patients

Pfeiffer I would not be surprised

Reifenstein You have the effect of one tropic hormone on the other tropic hormones

Here is another point When you induce a discharge of ACTH you get a decrease in the gonadotropic hormone You have for example a stressed animal The gonadotropic and also the thyrotropic hormone is decreased I am wondering if this relationship might make a decided difference here and if we are not dealing with an artificial situation that would not come about as far as the human is concerned

Asling I think any method to create single deficiencies would be good Whether there is some way of selectively affecting the pituitary s output of one hormone I do not know There are some indications that we might be able to get somewhere along that line in the next few years Single deficiency hormone studies are urgently needed When there is enough knowledge at hand they will be done

Copp We studied the effect on uptake of radioactive calcium in these animals

Asling I hoped you would discuss that

Copp We found very little effect of the ACTH in the normal animals There was a reduced skeletal uptake in the hypophysectomized animals treated with the hormone and from the radioautographs this appeared to be due chiefly to a lack of the sealing off bone in the treated animals In the hypophysectomized animals there was a very heavy deposit of radioactive calcium in this sealing off bone but this was absent in the experimental group This I think probably accounts for the difference I do not believe it is a fundamental effect on calcification

Bartter Did I understand you to say that the gonadotropins have no effect on bone maturation?

Asling No sir In the absence of the pituitary, estrogen and testosterone have not so far appeared to enhance bone maturation epiphyseal union and so forth This has been the experience of some other workers also I believe There are a few epiphyses which will mature such as a tiny center at the tip of the ischial tuberosities—some centers associated with the pelvis perhaps I should say And I must put in a major qualification from the work of Dr William R Lyons[151] who used testosterone alone and with growth hormone in male hypophysectomized rats The os penis is responsive to testosterone in its growth and not to growth hormone so that one may have by hypophysectomizing and treating with growth hormone an animal of full dimensions with a small phallus One may have with the converse (testosterone and no growth hormone) a very small animal with a more fully developed phallus including the ossification of its os Both hormones are needed to restore the balance

Shorr I think what is in back of Dr Bartter s mind is this that clinically one sees instances of profound growth disturbance go on to sexual maturity with closed epiphyses I wonder whether in these cases there are not small amounts of growth hormone, and that these small amounts of growth hormone might be sufficient together with the sexual hormones to produce maturation

Asling My understanding of this from some who have worked with growth hormone clinically is that the more recent highly purified preparations have not seemed so efficacious in human growth stimulation

Shorr I think it is too early to come to any conclusions about growth hormone in the human

Asling At present the best growth stimulus for human beings—perhaps someone will correct me—is a combination of testosterone and thyroxin

Armstrong Gentlemen we really must proceed

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151 Lyons W R Abernathy E and Gropper M Effects of Androgen and Somatotrophin on the Os Penis of the Rat *Proc Soc Exper Biol and Med* 73 193-197 (1950)

THE NATURE OF BONE AND PHOSPHATE ROCK

STERLING B HENDRICKS and W L HILL

From the Bureau of Plant Industry Soils and Agricultural Engineering U S Department of Agriculture Beltsville Maryland

Armstrong There are many facets to bone among which are several in the chemical field I think that we should go along to these now

Some of you remember that at last year's Conference I reviewed [132] as far as possible at that time our information on the constitution of the mineral phase of calcified tissues Since that time Dr Hendricks has carried the development of this subject very much further Through his courtesy I had the opportunity of seeing a manuscript prepared by Dr Hendricks before publication [133] Dr Hendricks has very kindly offered to bring us up-to date on this subject and show to us the most recent developments in the investigation of the mineral phase of calcified tissues

Will you go ahead Dr Hendricks?

Hendricks An explanation of my connection with the subject is required I have been interested in it for about twenty years the approach being that of a crystallographer I shall however not burden you with the special nomenclature of crystallography as I have been with that of histology

Dr Armstrong at these meetings last year presented the current aspect of the subject [132] I am glad that I was not present because I would have agreed completely with the subject as then summarized I did not know differently It was roughly at that time that the American Chemical Society decided to hold a symposium in Detroit honoring Dr Mack for her work and I was asked to summarize knowledge about the composition of the bone salt I was sure that the subject had been settled in much the way that Dr Armstrong indicated However after having left it for some eight years I suddenly realized with a fresh point of view that three or four small but extremely pertinent facts forced one to a rather diametrically opposite conclusion to that previously given

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[132] Armstrong W D Composition and Crystal Structure of the Bone Salt *Trans Macy Conference on Metabolic Interrelations* 2 11 31 (Jan 9 10 1950)

[133] Hendricks S B and Hill W L The Nature of Bone and Phosphate Rock *Proc Nat Academy Sci* 36 731 737 (December 1950)

Current Concepts of the Bone Salt

Two of the workers mentioned by Dr Armstrong were Dallemagne and Brasseur,[154-156] who have expressed what might be called the currently accepted conception of the bone salt I visited Dallemagne and Brasseur this summer and, in the course of two days' argumentation and discussion with them, attempted to show that a conclusion that they had reached which was similar to conclusions that we had reached independently in 1942 (the independence due to the lack of communication during the war) was *wrong* while indeed all of the facts that they had used were correct This also holds for the statement Dr Armstrong made about the work of Cartier[157]—this is on page 30 of the proceedings for last year—who concluded that bone is tricalcium phosphate, calcium carbonate, calcium citrate, and so on I doubt if bone is such material

Many people have expressed ideas on this subject, and of those I have seen I think that the ones expressed by Dr Hodge[158] are correct Having complimented him, I will now say that I think they were correct for possibly the wrong reason

Dr Armstrong, on page 12 of last year's proceedings, summarized the various ideas about what bone salt is, and here in Table XXIV they are

TABLE XXIV

Suggested Forms of the "Bone Salt"

1	Hydroxyapatite + $CaCO_3$ + other phosphate $Ca_{10}(PO_4)_6(OH)_2$
2	A carbonate apatite
3	Tricalcium phosphate hydrate containing carbonate

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154 Dallemagne, M J, and Brasseur, H The Diffraction of X-rays by the Mineral Substance of Bone *Bull Soc Royale des Sci de Liege* 11 451 and 488 (1942)

155 Dallemagne M J The Chemical Nature of Salts from Bones and Teeth and of Tricalcium Phosphate Precipitates *Nature* 157 453-455 (1946)

156 Dallemagne M J, Brasseur, H, and Melon, J The Constitution of the Mineral Substance of Bone and the Synthesis of Apatites *Bull Soc Chim* 16 109 (1949)

157 Cartier, P The Mineral Constituents of Calcified Tissues II The Molecular Structure of the Salt of Bone, *Bull Soc chim biol* 30 73 (1948)

158 Hodge, H C Some *Observations on the Dynamics of Calcification*, *Trans Macy Conference on Metabolic Interrelations* 2 73 112 (Jan 9 10, 1950)

summarized even more briefly It is an apatite there is no doubt about that this being the one definite result of x ray diffraction It contains carbonate and other minor constituents

People were forced into these rather different conclusions for quite simple reasons Dr Hodge[158] Dr Bale[159] Tromel[160] and perhaps other earlier workers reached the conclusion that the material is hydroxy apatite They compared the diffraction pattern of enamel which gives a relatively good diffraction pattern and that of dentin with the diffraction pattern of a specimen or preparation of hydroxy apatite They found no detectable difference so concluded that the material is hydroxy apatite That conclusion is correct

The Nature of the Carbonate

What might be the nature of the carbonate? They just said Well it is there That was practically the summary 'It is just around the place but they doubted that it was there as calcium carbonate Later Brasseur and Dallemagne in working on the carbonate said It is in bone as a crystalline calcium carbonate namely 4 or 5 per cent which we cannot detect because our methods are not that sensitive That is quite wrong The methods are sensitive enough to detect 1 per cent carbonate including the ones used by Brasseur and Dallemagne Moreover calcium carbonate can be detected in the order of hundredths of a per cent in virtue of the fact that it has a very high birefringence

Dr Armstrong and many others including me and my coworker Mr Hill have held that bone salt is a carbonate apatite similar to the mineral francolite This mineral can be obtained as crystals a millimeter or more in cross section They contain about 3 per cent carbon dioxide and are not essentially different from the bone salt except that they contain fluorine

If a structural crystallographer is told that a material contains 3 per cent carbon dioxide and that it forms a large uniform homogeneous crystal unless he has very good reasons for doing otherwise he has to accept the conclusion that carbon dioxide is part of the crystal part of the lattice as it were The acceptance of this conclusion has done more to confuse the question of what is the nature of the bone salt than any other thing The acceptance is not necessary As it will turn out the carbonate is not in the lattice even though the crystal is large and apparently uniform

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[159]Bale W F A Comparative Roentgen Ray Diffraction Study of Several Natural Apatites and Apatite-like Constituents of Bone and Tooth Substance *Am J Roentgenol* 43 735 (1940)

[160]Klement R and Tromel G Hydroxylapatite the Essential Part of the Inorganic Bone and Tooth Substance *Ztschr Physiol Chem* 213 263 (1943)

The Nature of Tricalcium Phosphate Hydrate

The next element of confusion concerns the nature of tricalcium phosphate hydrate We have ostensibly prepared this substance in crystals having dimensions of the order of 1 micron One can prepare the material in a wet way without much difficulty which we did as early as 1932 It gives the hydroxy apatite diffraction pattern and well might be hydroxy apatite Quite independently of us workers on fertilizer in Leningrad synthetized the material and came to the same conclusions about the formula Brasseur and Dallemagne also quite independently of us obtained this substance They said This is the material that characterizes the bone salt It differs from hydroxy apatite in that water is lost *upon heating* with formation of anhydrous tricalcium phosphate Hydroxy apatite still holds on to water at 1000 degrees but by 600 degrees tricalcium phosphate hydrate has lost all its water and has become a quite distinct and easily recognizable compound

Brasseur and Dallemagne said that the bone salt is tricalcium phosphate hydrate with calcium carbonate as an adventitious material They consider the carbonate to be present as a separate material because it can be preferentially removed by solution in acid After removal of carbonate ignition of bone gives anhydrous tricalcium phosphate I think that the conclusion is wrong

Brasseur and Dallemagne concluded that enamel is a carbonate apatite because upon heating it does not change to anhydrous tricalcium phosphate This conclusion is apparently supported by lack of preferential solution of the carbonate in acid They had apparent evidence that the material in enamel is a carbonate apatite and that the material in bone is a tricalcium *phosphate hydrate*

The next thing to realize about bone and enamel is that bone is essentially a neutral compound whereas enamel is more basic (see Table XXV) *Look first at enamel The numbers show the atom contents of the particular* element not percentages The positive ions are chiefly calcium with some magnesium and sodium The base of reference is total charge 20 The sum of the negative ions that are present ignoring for a moment citrate and some things like that is less than 20 by roughly two hydroxyl groups So it would seem that enamel if all of the accessory ions are correctly taken into account is indeed a basic material which in agreement with Hodge and others would be a hydroxy apatite *Bone on the other hand is essen*tially a neutral compound The total number of positive and negative ions without bringing in any hydroxyl whatsoever balances up quite exactly *So it would seem indeed* on the basis of that evidence to be a tricalcium phosphate ostensibly tricalcium phosphate hydrate and not hydroxy apatite

TABLE XXV

Typical Composition of Bone and Enamel

		Bone			
Ca		19.22	PO_4		17.18
Mg		0.56	CO_3		2.79
Na		0.22			
	Total	20.00		Total	19.97
		Enamel			
Ca		19.51	PO_4		17.50
Mg		0.38	CO_3		0.92
Na		0.11			
	Total	20.00		Total	18.42

Changes in the Surface with Grinding

It was this evidence (see Table XXVI) in part that suddenly caused us to wake up — this and the evidence in the case of bone that calcium carbonate can be preferentially dissolved out by the use of acid. The fact that one can preferentially dissolve the calcium carbonate of bone in acid is incontrovertible evidence that the carbonate is external to the apatite lattice. If you take a substance such as bone and grind to change the particle size of the material from which most of the organic matter has been removed—there could be a long discussion of how you remove this organic matter but in this particular case let us assume that the removal did no damage—you will find that as mesh size decreases the surface area of the material is unchanged as measured by the Brunauer Emmett Teller[161] method of nitrogen adsorption on the solid. It is of the order of about 80 m^2/g. Enamel on the other hand ground to pass 120 mesh has twice the surface area of 60 mesh material. A material of this mesh size—if it is not a spongelike material—would have a surface area of the order of only a few hundredths of a square meter per gram. Something has happened to change the surface.

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[161]Brunauer, S. *The Adsorption of Gases and Vapors I Physical Adsorption*, Princeton Univ. Press, Princeton (1943)

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[161]Brunauer, S. *The Adsorption of Gases and Vapors. I. Physical Adsorption.* Princeton Univ. Press, Princeton (1945).

TABLE XXVI

Change of Surface with Grinding of Bone Enamel and Francolite

Substance	Fineness	Surface m²/gm
Steamed Bone	*100 200*	66 2
	16 30	66 2
	4 8	63 7
Enamel	Through 325	6 8
	200 325	*3 7*
	60 120	2 1
Francolite	Below 6 microns	7 3
	100 200	3 2
	20 35	*1 6*

The mineral francolite which is the typical carbonate apatite also shows this behavior on grinding Upon passing from 20 mesh down to the order of 6 microns the surface area increases by four to five fold The explanation of what happens is in Figure 50 It seems that in the case of *bone the material has realized its ultimate surface and—I think this is im*portant for the explanation — there are no entrapped surfaces Enamel probably has about the same inherent surface as that of bone *although* physical measurements as have been made show that it is of the order of *2 to 7* m²/g instead of *80* m²/g The crystal is *full* of channels which are of the order of atomic dimensions They are of the order of twice the dimensions of a carbonate group and apparently they are not all interconnected so as to be available to a nitrogen molecule coming from an external surface such as that of a dry powder *Only* when the crystal has been broken do you find that these internal cavities can be reached In other words this carbonate apatite francolite upon which the whole concept was based—this single crystal—is a material that has many voids Moreover francolite upon crushing begins to show the preferential solution that is required if the carbonate is external to the lattice

The Nature of the Fluorine

Another fact which had caused us trouble for twenty years and for which we can now see the explanation is that francolite and phosphate rocks often contain fluorine in excess of that permitted by the formula of fluorapatite In the formula of apatite *the ratio of fluorine to 6 phosphorus*

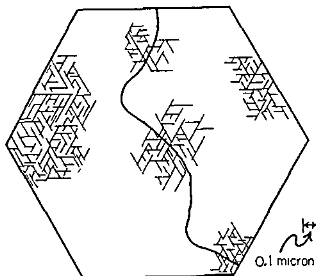


Fig 50 Schematic View of Entrapped Surfaces in Carbonate Apatites as Present in Enamel and Francolite

Reproduced with permission from Hendricks S B and Hill W L The Nature of Bone and Phosphate Rock *Proc Nat Acad Sci* 36 731 737 (December 1950)

atoms is exactly 2 You can analyze all the crystalline apatites in the world and find that they do not significantly go above 2 However in phosphate rock which is a material deposited by precipitation reaction, the ratio sometimes is as great as 3—in other words there are apparently 3 fluorine atoms in the formula instead of 2

Figure 51, which shows one unit of structure in a very simplified way indicates a possible condition in a small crystal The two fluorine atoms indicated by the arrow are the starting point for another unit of the lattice, as also are the other four fluorine atoms shown as block dots This is more fully shown in the larger figure If the crystal contains only one unit, these positions are available for occupancy so that eight instead of two fluorine atoms are present In other words, the composition of a crystal depends upon its size

Moreover, one can now go back to the phosphate rock and, from its excess fluorine, calculate the size of the crystal since the excess fluorine can only be on the faces The result for the surface area of the phosphate rock is 60 square meters per gram If now it is assumed that bone has realized the ultimate particle size, namely, the order of 60 to 100 square meters per gram and that carbonate completely covers the surface, an estimate can be made of the area required for a carbonate group The carbonate content of rock then leads to an estimate of surface which in this case is 60 m^2/g In other words, the inherent particle size of phosphate rock in this case can be calculated from the carbonate content of bone

There is one other strange fact about phosphate rock that has a bearing on the nature of bone Phosphate rock contains two principal minerals one known as colophane and the other as francolite Under the petrographic microscope colophane has a very low birefringence, while that of francolite is moderate It has been noticed that a small amount of CaF_2 often appears as the mineral fluorite in those rocks containing francolite The amount of fluorite, however, is generally small Geologically speaking there was no reason for its restricted formation The answer, apparently, is that the colophane has a larger internal surface than does francolite The larger crystal grew at the expense of the smaller crystal and it could only do so by wringing fluorine off the surface and having it appear as calcium fluoride That did indeed happen

Finally then it is known that fluorine and carbonate can be on the surface, and that enamel does not differ from bone in any essential structural way It is a more basic material, apparently, but it too contains carbonate on the surface This surface, however, in enamel is an internal one It is much as if the crystals grew until their faces were largely covered with carbonate, citrate and other ions, then additional apatite material formed over the surface it in turn being limited in growth by surface reactions

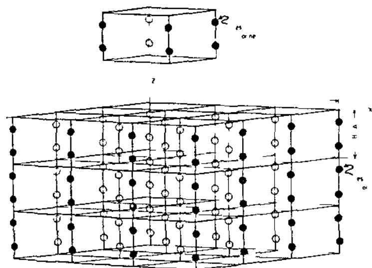


Fig. 51 Excess Fluorine in an Apatite type Crystal due to Lattice Limitation

Reproduced with permission from Hendricks, S. B. and Hill, W. L. The Nature of Bone and Phosphate Rock. *Proc. Nat. Acad. Sc.* 36 731-737 (December 1950)

The Nature of the Citrate

Just at the time when citrate was found as an essential constituent Dr Armstrong and I were having a discussion and he raised the question Why not assume that citrate is in the lattice? That was a crystallographer s nightmare We were quite willing to assume that carbonate was but when you come to a big molecule like citrate it just will not fit The answer is that the citrate is characteristically on the surfaces of these hydroxyl apatites

The Nature of the Magnesium

We were in for a surprise Very old work on bone had shown that *magnesium has preferential solubility and the very excellent work* of Logan and Taylor[16] which was published in 1938—and if one had followed it one would have fallen into fewer traps—showed also that sodium is on the surface

Magnesium is an ion which would perfectly freely have been accepted as going in place of calcium into the lattice It was a surprise indeed to find that the magnesium is external to the crystal The reason is a rather interesting one The magnesium ion in solutions near neutrality does not exist as Mg^{++} which is the cation isomeric with Ca^{++} Instead it is solely there as the $(MgOH)^{+}$ cation which is entirely different from Ca^{++} and *apparently is excluded from replacement of calcium in the lattice* Sodium differs from Ca^{++} while having the same size it cannot go into the lattice because of difference in charge

One of the more interesting surface ions is uranyl It is permissible to tell you that one of the principal uranium sources in the United States is phosphate rock due to the fact that phosphate material characteristically takes up the uranyl radical upon its surface

The Nature of the Bone Salt

One final point is to be made What about the fact that bone has the composition of a tricalcium phosphate not of a hydroxy apatite and still is a hydroxy apatite!! That has to do with the likelihood that bone is hydroxy apatite with excess phosphate upon the surface The excess phosphate is not just the right amount for a neutral salt since the composition does not exactly equal that of tricalcium phosphate This interpretation of the role of surface phosphate still has to be verified

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[16] Logan M A and Taylor H L Partial Solution of Bone and Carbonate Containing Calcium Phosphate Precipitates *J Biol Chem* 125 391 (1938)

bone you see is forming in the presence of carbonate in a system in which the magnesium ion is $MgOH^+$ and in which the phosphate is $(HPO_4)^{=}$. Apparently what happens is the following. The crystal starts growing and as it grows as hydroxy apatite it has got a trashpile of stuff that it does not want in the hydroxy lattice and it starts excluding these. Among these ions is carbonate, magnesium and sodium. As the crystal grows these materials apparently completely cover the surface and stop the growth. So one characteristic of bone salt is that it is prevented by the mere adventitious materials that get upon the surface from growing beyond a certain size. A new surface can be started by covering up this old one and this is done in the case of enamel. A new surface can also be started just by growing as an absolutely independent crystal and that apparently is done in the case of bone.

Conference Discussion

Armstrong: Thank you, sir. It has been extremely interesting to every one. It shows us how quickly the Minutes of our meetings can grow out of date.

Hendricks: Well, at least minutes and not seconds.

Hastings: May I be the first to congratulate Dr. Hendricks, who was sort of an old hand in this field before we knew anything about it. We got off on the carbonate apatite theory and it is a great pleasure to hear such a clean-cut presentation of the situation.

I should like to get this one point clear. I take it that those spots which were indicated as the positions for fluorine on the surface of the crystal are the only spots where the CO_2 can appear.

Hendricks: No, I think it is much simpler than that, Dr. Hastings.

I cannot see the detail of the way in which the carbon dioxide is upon the surface nor can I see the detail of the way in which the citrate is upon the surface. However, they definitely are not associated with the fluoride positions. You can fill the fluoride positions in phosphate rock and still have the carbonate there, or rather the carbonate seems to cover the whole surface.

The general rule about it is the following: that any substance that will form an insoluble calcium salt or will sequester calcium salt apparently will form a surface compound with bone.

Hodge: Will you say that again please?

Hendricks: Any substance that will form an insoluble calcium salt or will sequester calcium, as does citrate, will form a surface compound with bone.

Neuman: I will underwrite that last statement. At least, it certainly looks that way.

I feel moved to bring up a suggestion that was made by Robert Kunin and then also to stick my neck out a little on one of the possible means of explaining the difference between the surface lattice positions of hydroxy and fluoride and the surface covering.

Robert Kunin[163]—I have been advertising his work—has spent years running around to Government offices but as far as I know his study has not been released for publication. He prepared a whole series of phosphate compounds carefully controlling the pH and his conclusion was that there is a limited amount of substitution possible in the apatite lattice as follows: that if we write the conventional formula



$$Ca^{++}_{10-x}\,(H_3O^+)_{2x}\,(PO_4)_6\,(OH)_2\,(C_{10}PO_4H_2),$$

some substitution of calcium may take place. Ca+ can vary from 8 to 10, and the hydronium ion then would vary from 0 to 4. If there is maximum substitution of hydronium ion and you heat the bone, this hydronium ion will dehydrate at about 400 degrees. When you do that, you see your lattice configuration should collapse. When you have maximum substitution and the material is formed at a low pH, a relatively low pH, then the lattice is not stable to heat because of the dehydration of the hydronium ion at 400 degrees and it collapses giving β calcium phosphate.

Hodge[164] and coworkers and Sobel *et al.*[165] put this same phenomenon *in terms of the calcium phosphate ratio. If the phosphate* was high relative to calcium, the material was not stable to heat and converted to tricalcium phosphate, as you have mentioned.

Hendricks: I would agree with the latter statement, not that of Kunin.

Neuman: I'm not sure there's a difference in the final analysis.

The amount of water lost at 400 degrees, which incidentally, is the theoretical temperature for dehydration of the hydronium ion, is related

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[163] Kunin, R., Elmore, K. L., and Johnson, D. L.: Basic Region of the System Calcium Oxide-Phosphorus Pentoxide-Water. (in press).

[164] Hodge, H. C., LeFevre, M. L., and Bale, W. F.: Chemical and X-ray Diffraction Studies of Calcium Phosphates. *Ind. and Eng. Chem.* 10: 156-161 (1938).

[165] Sobel, A. E., Hanok, A., Kirshner, H. A., and Fankuchen, I.: Calcification of Teeth. III. X-ray Diffraction Patterns in Relation to Changes in Composition. *J. Biol. Chem.* 179: 205-210 (1949).

to the ratio of calcium to phosphate assuming this substitution does take place One can calculate a theoretical line The points for some thirty five precipitates fell right along it very, very nicely Kunin is not the originator of this idea but I think he has the best evidence to support it It must be considered

I'd like also to discuss the problem of surface ion binding We started to study CO_3 and then got involved in some technical problems It became apparent that there is a hydration layer on the apatite crystal which may have a bearing for the surface-bound layer

Most crystals whatever their shape do not possess a complete lattice at the surfaces but are relatively rich in one or the other principal constituent If for the moment you assume that the surface is relatively rich in calcium and you plot the potential due to the localization of the positive charge as you go into the interior of the crystal the potential will fall You will get to a perfectly normal crystal only where no net charge is observed This potential would have to be matched by an equal concentration of negative charges in the solution phase This is essentially a double layer effect

If we assume then that this crystal's surface is relatively rich in calcium then there has to be held in the surface immediately surrounding the crystal an equal number of negative anions and I believe when bone are subjected to drying the ions in this hydration layer would not be removed The ions would stay there Only the water would be removed

You would analyze these hydration layer ions as part of the total structure but in water, these are not an integral part of the crystal In this hydration layer would be fluoride phosphate bicarbonate and the like or carbonate I do not know which

Armstrong That would require a ridiculously high concentration of fluoride in the fluid

Neuman No it would depend on the relative affinity of these ions for calcium as Dr Hendricks pointed out I do not know what all would be involved It would be a rather complicated thing It would have to be determined, I think by experiment

I should like to hear your comments Dr Hendricks What do you think of something like that?

Hendricks I think honest and straight forward discussion is the best It seems to me both points you raise are confusing I believe that it is a much simpler system than the one you mention that the apatite is simply growing like any other crystal just like sodium chloride for instance except that apatite peculiarly forms compounds with its surface and that

is not at all the classical boundary phenomenon The classical boundary phenomenon we see very well exemplified in clays and base exchange materials This does not seem to be a material like that It seems to be a material whose surface can very easily form a compound There is calcium on the surface The calcium is not necessarily a part of the lattice But on that surface it can form a compound with carbonate with citrate or anything like that

I should like to keep the picture in its simplest aspect until forced into greater complexity because the complexities that have been added have confused the issue for all of these long long years

One finding of Logan and Taylor[16] should be pointed out They observed that if you precipitate a calcium phosphate *in vitro* from a solution having about serum which contains carbonate and so forth the material which first came out was essentially carbonate free and then standing in contact with the solution it took up carbonate and gave up phosphate to the solution as if the phosphate itself were coming off the surface and being replaced by carbonate This apparently would be a process that did not inself involve the apatite lattice but involved the surface phosphate on the apatite lattice

Kunin s evidence for the hydrate is still very slim It is very difficult to demonstrate as to whether the material is a tricalcium phosphate hydrate or whether it is phosphate on the crystal I think the best method of doing this would be by exchange experiments with P^{3}

The presence of these cracks has an important bearing on several questions such as the relationship of fluoride to teeth Apparently fluoride migrates far more readily than does many other substances and will get over quite a large amount of that total surface

Armstrong Last summer Brasseur showed me a film and I imagine he showed the same film to you I was willing to accept the fact that the film showed the presence of crystals of calcium carbonate in a specimen of bone Would the type of association of calcium carbonate which you have described with the fundamental calcium phosphate be expected to show the X ray diffraction of calcium carbonate?

Hendricks No it would not show at all the diffraction pattern of calcium carbonate

I might mention here that this has been discussed with Dr McConnell with whom you worked and it has been like pulling teeth from him to accept any of it He brings up this point that francolite is biaxial, it is not uniaxial like apatite It is not a hexagonal crystal in other words And the answer to that is that with carbonate groups which are themselves

inherently highly birefringent on the surface you can do anything with the axis that you wish but the face is not sufficiently sensitive to give anything other than the hydroxy apatite pattern

If Brasseur and Dallemagne really found calcium carbonate it is there I do not have an adequate enough knowledge of histology to know whether or not there are conditions of bone under which calcium carbonate actually deposits as such

There is another interesting point here We have worried about what would be the stable phase in a calcium P_2O_5 water system Many people have tried to make it, among them Dr Hastings We think that the stable phase may not be an apatite at all but rather anhydrous tricalcium phosphate

It is interesting that in the body anhydrous tricalcium phosphate does occur at times as urinary calculi—if that is sufficiently technical

Hastings You are learning fast

Hendricks It might be the stable apatite but it is prevented from forming in most of the body systems

We have found one other place a geological place in which it is a deposit from bat guano

But there is evidence that there is truly a calcium phosphate bone In other words bone is always a metastable system

Shorr May I ask Dr Hendricks what is the nature of the strontium salt that is deposited?

Hendricks It could be straight apatite Strontium would go identically with calcium And strontium moreover does not have that objection of magnesium namely being there dominant as a basic ion Strontium can replace calcium as such.

Sobel I have been in correspondence for the last year and a half with a young man named T G Taylor from the University of Reading in England He has been doing studies with our pet theories which are in conflict with some of your findings if we accept his studies which appear to have been done very carefully He took actual bone rather than ashed bone on which most of such experiments have been performed and very carefully studied what went into solution at various pH s and what was left behind He also added to those studies enamel and dentin Much to my surprise and to his own the proportion of the components (calcium magnesium phosphate citrate and carbonate) that went into solution at various pH s was indentical to that which the solid itself had

In view of the statement that these compounds on the surface were derived essentially from experimental data indicating that these go preferentially into solution if Taylor s experiments can be confirmed — and I think his experiments are all right — we might have to think about the subject matter again

I do not have his charts with me as I did not anticipate this topic but next year it might be worth while to bring up Taylor s experiments I suppose by then he will have published them

Hendricks Did I understand that the statement included carbonate?

Sobel Carbonate citrate magnesium calcium and phosphate

Hendricks Against that a half dozen workers have specifically shown that carbonate preferentially dissolves

Sobel Most of those studies have been done on ashed bone

Let me describe his experimental method

He takes some ground up bone puts it in a bottle and leaves it there for about 24 to 48 hours And the end of that time he analyzes the supernatant fluid More recently he added to that the analysis of the residue in the flask

Most other experiments have been repeated extractions rather than the type of equilibrium which he has carried out

Hendricks There is of course the grave objection to that particular type of experiment of not dealing with the material from which the organic matter has been removed I think some of you will readily see that materials like collagen also could form surface compounds with bone and that these materials might just so exclude the surface of the materials as to force the equilibration in Taylor s work to be by complete resolution of bone

McLean Dr Hendricks I wonder whether you will tell us when and where this will first appear in print

Hendricks It is in the *Proceedings of the National Academy*[153]

Hastings Would you repeat for me what you think the size of the hydroxy apatite crystal is before it starts being a surface

Hendricks In the case of francolite it is calculated to be 2[illegible] of structure The difficulty is this I do not know the relative ex[illegible] the crystal in the direction of the principal crystallographic a[illegible] case of bone it comes out to be about 10 units of structure [illegible] the order of 100 angstrom units in inherent particle size

I should like to return to Taylor's work which bears the further implication that citrate is characteristically present on the surface I mean citrate is too imbedded in the material and dissolves as such and you cannot place citrate anywhere in the lattice Citrate cannot be inside the actual crystal structure

Johnson Perhaps this might be the time to inject for the thought of everyone some conversation that went on over cokes and our dinner yesterday among a few of us which depends upon the eventual synthesis of the work that Dr Hendricks has done and Dr Bloom's work and Dr Hastings' experiments which he talked about yesterday The general tenor of the conversation was this that the very rapid turnover of the 10 minute transfusions which Dr Hastings did primarily took out of the bone possibly, the surface component but did not get at the basic crystal Bloom's work using astronomical doses of parathyroid hormone went beyond that, but it is important that in his work there was no osteoclast activity in that first phase The absence of osteoclast activity may be associated with the loss of the surface adsorbed material while its presence is associated with the basic crystal loss as the matrix is destroyed

That is why I was going to get at some method of determining what the salt change is surrounding the lacunae and preceding the appearance of its osteoclasts in the question yesterday afternoon Whatever the percentage of calcium that is surface bound let us say 12% it appears that halisteresis may be 12% correct—88% wrong! That is a paraphrase of the situation

Armstrong It is contrary to my nature to urge people to close down on discussion but I am constantly faced with the problem of seeing that we have served to us all of the intellectual food that we have planned for the table

SOME CONSIDERATIONS OF THE SOLUBILITY OF CALCIUM PHOSPHATE[166]

HAROLD C DODGE

From the Division of Pharmacology and Toxicology, Department of Radiation Biology School of Medicine and Dentistry University of Rochester Rochester, New York

Armstrong Dr Hodge will now discuss his recent studies on the solubility of the bone salt

Hodge A continued search of the literature has to date provided a list of less than two dozen investigations in which analyses have been made of calcium and phosphorus concentrations in the solutions above precipitates of basic calcium phosphates In some of these studies large excesses of calcium or of phosphorus were added to the solutions intentionally Sometimes by plan and sometimes inadvertently the time allowed for equilibrium conditions to be established frequently may have been inadequate, although it is possible that the marked deviations from the solubility curves shown in Figure 52 are to be attributed to the presence of relatively stable soluble complexes, rather than a lack of conditions approaching equilibrium Not all of the analyses agreed amongst themselves as well as those shown in Figure 52, a complete presentation of all the available data would be so overburdened with points that the picture would be hopelessly confused

Starting with the work of Bassett (1908)[167] and Cameron and Seidel[168] and including some of the data of Holt La Mer and Chown,[169] Sendroy and Hastings[170] the current work of Hodge,[171] together with the recent work of Elmore and Farr,[172] an average line was drawn (see the lower line

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[166]*This paper is based in part on work carried out under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester New York*

[167]Bassett H Jr Beitrage sum Studium der Calciumphosphate *Z anorg Chem* ***59*** *1 55 (1908)*

[168]Cameron F K and Seidell A Phosphates of Calcium *J Am Chem Soc* **26** 1454 1463 (1904)

[169]*Holt L E Jr La Mer V K and Chown, H B* Studies in Calcification *J Biol Chem* **64** 509 578 (1925)

[170]Sendroy J Jr and Hastings A B Studies of the Solubility of Calcium Salts (II The Solubility of Tertiary Calcium Phosphate in Salt Solutions and Biological Fluids) *J Biol Chem* ***71*** *783 796 (1927)*

[171]Hodge H C unpublished results

[172]Elmore K L and Farr T D Equilibrium in the System Calcium Oxide Phosphorus Pentoxide Water *Ind and Eng Chem (Industrial Edition)* **32** 580 586 (1940)

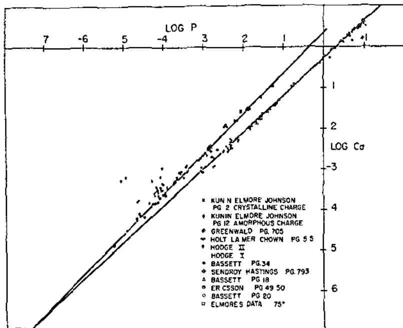


Fig 52 The Concentration of Calcium Plotted Against That of Phosphorus

Calcium is the ordinate Both concentrations are plotted on logarithmic scales The data tend to fall along two trend lines as shown The lower line represents systems in which the calcium phosphate has precipitated and has been equilibrated for various lengths of time The upper line represents systems in which a precipitate has been resuspended in water with or without salts in solution

in Figure 52) This grouping was achieved from the similarity of the constants in the equations for the log Ca log P relationships derived independently for each study

At the same time that these data were plotted the data of Ericsson[173] and of Greenwald[174] were also plotted It was immediately apparent on inspection that the data of Ericsson and Greenwald did not fall on the same line as had been calculated for the data of the investigators listed above It appeared that Ericsson s and Greenwald s data closely approximated a straight line (see the upper line in Figure 52) placed above but having approximately the same slope as the lower line first drawn When the data of Kunin *et al*[175] were added these lay close to but slightly above the upper straight line

Separation of the Data into Two Groups

Some reason was sought for the separation of the data into the two groups as represented by the two lines in Figure 52 It was soon discovered that the systems lying along *the lower line* in every case had been prepared by adding solutions of phosphoric acid or some phosphate salt to solutions of calcium hydroxide or some calcium salt and then equilibrating the resultant suspension for various periods of time (weeks to months) In contrast along *the upper line* the analyses of Ericsson, of Greenwald and of Kunin *et al* were made on systems that had been prepared by adding varying amounts of dried solids to water or to aqueous solutions and equilibrating for various periods of time *Consequently the data on the* upper curve represent concentrations of calcium and phosphorus that have been achieved by a process involving solution of a solid whereas the data lying on the lower curve represent residual concentrations of calcium and phosphorus in solutions from which solids have been precipitated

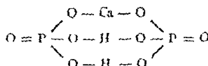
When the concentrations of calcium and phosphorus were expressed as mols per liter it was found that the slope of the curve for the precipitated systems is about 0 93 so that the relationship for the lower line is as follows $\frac{Ca}{P^{0\,93}} = 0\,4$ approximately The significance of the exponent of

[174]Greenwald I The Solubility of Calcium Phosphate *J Biol Chem* 143 703 714 (1942)

[175]Kunin R Elmore K L and *Johnson* D L Basic Region of the System Calcium Oxide Phosphorus Pentoxide-Water (In press)

the phosphorus concentration is unknown The equation for the line of the solid-added to solutions systems is as follows $\frac{Ca}{P}$ 1 3 approximately The simplicity of the calcium to phosphorus ratio i e the exponent of P is unity is an encouragement to seek an explanation for the relationship

In the case of the "precipitated" systems the calcium to phosphorus ratio is near enough to 1 2 to suggest that the principal anion is primary phosphate The constancy of calcium to phosphorus ratio over such a wide range of calcium and of phosphorus concentrations respectively may be due to the presence in the solution of a soluble complex of calcium phosphate perhaps having some such formula as



In the case of the "resuspended solid" systems the calcium to phosphorus ratio appears to be somewhere in the range of 1 3 to 2 1 If a crystal of the apatite lattice went into solution the expected calcium to phosphorus ratio would be about 1 7 Again the constancy of calcium to phosphorus ratio over such an enormous range of concentration over total dissolved substance suggests that some other soluble complex of calcium phosphate may be present in this case with a composition approximating the hydroxyl apatite empirical composition

There is one exception to the location of points according to the method of preparation of the suspension In a series of resuspensions of solid phosphates Sendroy and Hastings added various amounts of calcium and phosphorus to the solution in serum These points fall along a line which appears to be roughly parallel to the other two lines in Figure 52 but is placed below the curve for the precipitated systems

Calcium to Hydrogen Ratio

In Figure 53 are plotted the logarithms of the molar concentrations of calcium against the pH for most of the systems about which we have information It is apparent that despite a marked degree of variability the points appear to cluster about a line of slope unity It is probable that the amount of scatter increases with increasing dilution and increasing pH Unfortunately Bassett and Cameron and Elmore provided no record of the acidity of their solution However, Elmore and Farr have estimated for Bassett s data that the pH of the most dilute system in which secondary

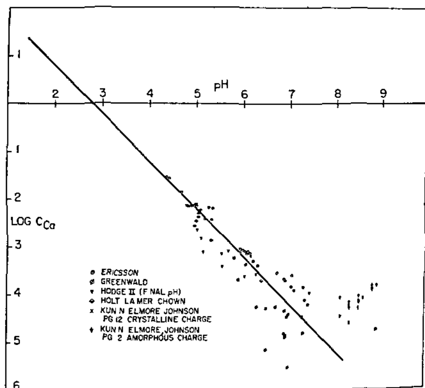


Fig 53 The Molar Concentration of Calcium Plotted Against pH

Calcium is the ordinate and the logarithm of the molar concentration is plotted Although there is marked scattering of the points there is a linear tendency

phosphate was the solid phase probably was about 2 8, this point does not lie precisely on the line but it differs no more than do points at the higher pH s The equation of the line would indicate that $\frac{Ca}{H} = 60$ approximately however the significance of this ratio is doubtful By selecting a dozen typical solutions all done by the same investigator variations of 40 to 6 000 were encountered although in the work of Holt La Mer and Chown and of Hodge values in the range of 400 to 600 were found The kind of variability that exists is well illustrated in the lower right hand part of the figure where the data of Kunin *et al* appear to describe a line running at approximately right angles to the indicated trend line

Log Calcium vs Log Phosphorus Minus pH

On the assumption that $\frac{Ca}{P} = k_1$ and that $\frac{Ca}{H} = k_2$ the combined equation should read $\frac{Ca^2}{H \times P} = k_3$ This can also be expressed as 2 log Ca=log P−pH+k_4

In Figure 54 the logarithm of the molar concentration of calcium has been plotted against the logarithm of the molar concentration of phosphorus minus the pH It is obvious that there is considerable scatter, it is also obvious that in general there is a linear tendency as would be predicted The trend line indicates at over a wide range of concentrations of calcium and phosphorus and over a considerable range of pH a single line can describe the relationship between these three quantities The single point referred to above that may be used from Bassett s work fell remarkably close to the trend line thereby making this relationship serve at least approximately over calcium concentrations varying from 10^{-1} to 10^{-5} M (4 log units) and over range of values of log P pH of no less than 11 log units

Variations with Time

In the experiments of Logan and Taylor,[176] analyses were performed at varying times after the initial mixing In some cases the pH was maintained nearly constant by additions of sodium hydroxide as needed It is interesting to observe that there was a uniform trend for the solutions to lose calcium and to lose phosphorus (because the pH was maintained al

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[176] Logan M A and Taylor H L Solubility of Bone Salt (II Factors Affecting its Formation) *J Biol Chem* 125 377-390 (1938)

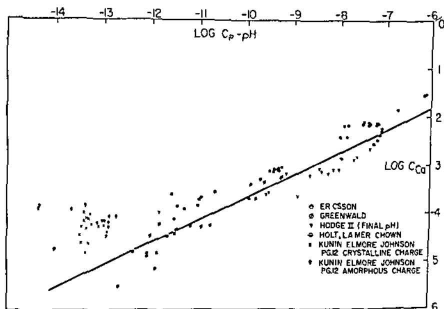


Fig 54 The Molar Concentration of Calcium Plotted Against the Molar Concentration of Phosphate Minus pH

Calcium is the ordinate and is plotted on a logarithmic scale against the logarithm of the molar concentration of phosphate minus pH Over a wide range of concentrations of calcium, phosphorus and hydrogen ions a linear tendency is found

most constant) in the early time periods. It is also interesting to find that the equilibrium concentrations were close to or approaching the trend line. The latter phases always involved additional decreases in calcium concentration along with increases in phosphorus concentration. In their experiments numbers 130 and 131 in the first hours a course is described which is roughly parallel to the course of the trend line but placed considerably above it. Such behavior suggests that in addition to calcium and phosphorus in solution in the concentrations expected from the trend line there was other "soluble" calcium phosphate. The approach of the curves in each case toward the trend line again suggests that in some way it may define at least roughly the equilibrium condition.

Calculus Formation

In the recently published work of Leung [17] the calcium and phosphorus concentrations of saliva samples shaken with solid synthetic hydroxylapatite have been measured at various CO_2 tensions. When the saliva was first collected calcium and phosphate values were such as to indicate the considerable supersaturation with respect to the trend line in Figure 54. Upon equilibrating with nitrogen containing no CO_2 the CO_2 from the saliva was almost entirely removed. Simultaneously a large part of the calcium and considerable phosphorus was thrown out of solution and the pH increased a little. The point now reached lay close to the trend line. Subsequent treatment of the saliva in contact with hydroxylapatite with nitrogen containing 5 to 22% CO_2 gave calcium, phosphorus and pH values such that a series of points was described lying close to the trend line although both the calcium and phosphorus contents increased several fold. This is an additional indication that the trend line defined the saturation conditions for hydroxylapatite.

From Leung's data the hypothesis may be advanced that the loss of CO_2 from the saliva in the mouth may be associated with the production of calculus on the teeth. Dental calculus as far as its mineral phase is concerned is hydroxylapatite. Conditions favoring a reduction in calcium and phosphate contents of saliva to values like those along the trend line for solid hydroxylapatite offer theoretically a partial explanation for calculus formation.

Solubility as a Function of Amount of Solid Phase

Greenwald in 1942 presented a convincing demonstration that increas

[17] Hodge H C and Leung S W Calculus Formation *J. Periodontol.* 21 211-221 (1950)

ing the amount of solid phase was associated with an increase in the amount of calcium and phosphate in solution His interpretation was that the ion product increases with the amount of solid phase Greenwald confirmed the work of Klement and Weber (1941)[178] in which amounts of precipitated hydroxylapatite dried and weighed varying from 100 to 1200 milligrams were equilibrated for short periods with water and the amounts of calcium and phosphorus in solution increased with increasing amounts of solid

We were greatly interested to find that an excellent approximation to a linear relationship is obtained from the data of Greenwald when the logarithm of the grams of solid phase is plotted against the logarithm of the calcium concentration in solution Whereupon we plotted the data of Kunin *et al* in a similar fashion and discovered that two straight lines were obtained one for the solutions above the amorphous solid and another for the systems in which the solids were crystalline These two lines were nearly parallel closely placed and had slopes considerably less than that of Greenwald's data The data of Klement and Weber agree reasonably well with the line for amorphous material of Kunin *et al* A practically parallel line placed somewhat above those of Kunin *et al* was found for some data furnished by Levinskas and Neuman[179] The data of Hodge although exhibiting considerable scatter may well also have a linear trend, the line in this case lies at still higher calcium concentrations The data of Ericsson show still more marked variability and lie at still higher calcium concentrations but may be said at least in part to fall near the lines for the data of Hodge and of Levinskas and Neuman

Dried solids were suspended in 0 1 N sodium chloride solution by Levinskas and Neuman presumably [illegible] water by Kunin *et al* in conductivity water and paraffined f[illegible] [illegible]nt and Wel[illegible]and in a variety of salt so[illegible] by Ericss[illegible] [illegible] washed it by [illegible] and kep[illegible] susp[illegible] for a year before [illegible] [illegible]xperi[illegible] [illegible] on were diluted [illegible] of [illegible] to [illegible] [illegible]ntra tion of 0 2 [illegible] des[illegible] [illegible]ig dilute ph[illegible] dil[illegible] [illegible]ably to one liter [illegible] temperat[illegible] extraord[illegible] increasing[illegible] calcium in[illegible]

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[178] Klement [illegible] 74B [illegible]

[illegible]evinskas [illegible]

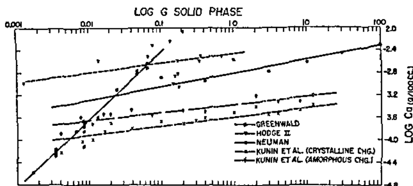


Fig 55 The Calcium Content Plotted Against the Total Amount of Solid Phase in a Liter of Suspension

Calcium in grams/100 cc is the ordinate Both items are plotted as logarithms The studies of Kunin *et al*[1] and of Levinskas and Neuman[9] are on resuspended precipitates The studies of Hodge[171] are on solutions in which precipitation occurred Greenwald[174] used aliquots of a suspension suitably diluted

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[178] Klement R and Weber R Behavior of Hydroxylapatite in Solutions *Ber chem ges* 74B 374 386 (1941)

[179] Levinskas G and Neuman, W unpublished results

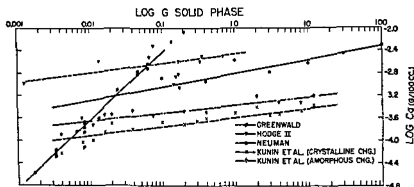


Fig 55 *The Calcium Content Plotted Against the Total Amount of* Solid Phase in a Liter of Suspension

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[178] Klement R and Weber R Behavior of Hydroxylapatite in Solutions *Ber chem ges* 74B 374 386 (1941)

[179] Levinskas G and Neuman, W unpublished results

Hodge As far as calcium ion is concerned?

Hastings Surely

Hodge I should like to ask Dr Hendricks what sort of picture he has of the gelatinous precipitate that can be prepared which has the theoretical calcium to phosphorus ratio but which will not give an x ray diffraction pattern

Hendricks It is a gelatinous precipitate which has the correct calcium phosphorus ratio in the hydroxyl apatite?

Hodge Yes sir Have you ever seen one of those?

Hendricks No I have never seen one

We have been attempting to prepare Warrington's octa calcium phosphate[180] recently It is material made by—the order is important but I am not sure of the order—dumping the large excess of calcium chloride into phosphate An amorphous material is obtained under such conditions Warrington made this material in 1872 and called it octa calcium phosphate It might be amorphous material thrown out very very quickly but still it does not have the composition of hydroxyl apatite

Hodge I have prepared an apatite other people have too in which there is a great deal of water up to 2000 per cent water with a calcium phosphorus ratio of about 2 1 which on drying and heating and igniting gives a hydroxyl apatite pattern but which just taken out as a little pat of jelly does not have any

Hendricks This is just a gel?

Hodge What I am getting at is this Do you suppose that that gel has in it some dehydrated separate units that are very much the form the hydroxyl apatite would ultimately take?

Hendricks There is no necessity to do so

Hodge I tried to formulate this thing somehow or other You might like to see how I tried to do it (see Figure 56) In the solution we would certainly have some kind of equilibrium At pH 7 there is mostly secondary phosphate This ion could react with calcium to form crystals of secondary calcium phosphate hydrate It could form one or more soluble calcium phosphate complexes For instance we might have one with a 1 2 Ca to PO_4 ratio one with a 10 6 ratio There may be a variety of

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[180] Warrington R Researches on the Phosphates of Calcium and Upon the Solubility of Tricalcic Phosphate *J Chem Soc (London)* 19 296 (1866)

of solution vary over a five fold range viz from 0 001 to 100 grams per liter Certainly the recognition of this dependence of solution concentration on the amount of solid phase is important In more than one case in the past investigators have failed to mention the amounts of solid phase used thereby making it impossible to compare their results with those of othe investigators The order of magnitude of change obtained in calcium con centration is roughly as follows a 1000 fold increase in the amount of solid phase is accompanied by a ten fold increase in calcium concentration The form of the soluble calcium is unknown again the possibility of some sort of soluble calcium complex may be suggested

Conference Discussion

Hodge The inconsistency is a thousandfold The inconsistency of this product varies The pK runs from—where do you want to start? Twenty three somebody says Values up to 33 are in the literature There is a range of 11 log units I do not see how it can be used

Bartter When you say that the pK varies 11 points between what do you mean? One animal and another?

Hodge Between different experimenters For example take almost any single paper go back to the work of Hastings take Logan and Taylor s work Greenwald s work take my own work Just pick data out of the literature If you multiply the cube of calcium concentration times the square of phosphate the values run all the way from 10^{-24} to 10^{-33} LaMer *is a good physical chemist but when he got a constant that ranged from* 10^{-24} to 10^{-33} he was abashed He did not even put that 10^{-33} in his table he left that space blank

Hendricks Is that PO_4^{3-}?

Hodge Yes

Armstrong You are going too fast for me What is the power to which you raised the phosphorus?

Hodge 0 93 the slope of the lower curve in Figure 52 I do not know what the 0 93 means

Hastings You just have salt solutions? You do not have any plasma figures do you? That would make a big difference as to how you are plotting the data

Hodge There was at least one study in which plasma was used I have just total calcium

Hastings If they are just total calcium concentrations then those data are wrong

these complexes—soluble complexes The ions can also form the hydroxyl apatite lattice

You see, the presence of the soluble complex rather than ions of calcium and of phosphate in the solution may be the thing that controls the calcium to phosphorus ratio in the solution over this enormous range of concentration All we have to assume is that there is not very much calcium or phosphate as ions and a potentiality of a great deal of calcium and phosphate as a soluble complex, and we have it

Hastings Dr Hodge you know that Logan and Taylor[181] and Logan and some of the other fellows really did a pretty thorough investigation of the effect of different amounts of solid We had done a few preliminary experiments before that Regardless of whether they calculated them as pK s for tertiary calcium phosphate I would think that for constant phosphate and constant pH their results would show a decrease of the calcium in solution as a function of amount of solid

That was done both with ground bone salt and with solid phases of artificially prepared hydroxy apatite Isn t that contrary to what you showed in that last chart?

Hodge That is right

Hastings How would you account for that?

Hodge From their data you have to decide exactly the reverse that the more solid phase present the more calcium there was in solution I think there are at least two things that can be said (1) I recalculated their data and found that the amount of solid phase changed during their suspension period by an important amount not by a little amount This was in the data as presented *in toto* but they did not make the calculation and I am not sure they took this change into consideration This takes a lot of the apparent disagreement away

The second one is that I am almost sure that their suspensions had not yet had time to get to equilibrium

Hastings They were

Hodge The data for some of their suspensions the 30 day ones for instance when plotted on the log Ca *vs* log P—pH graph gave a series of points In the first few hours there was a rapid change in the direction

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[181] Logan M A and Taylor H L Solubility of Bone Salt *J Biol Chem* **119** 293 (1937)

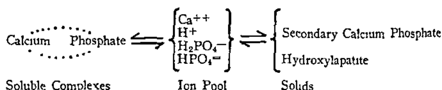


Fig 56 Formulation to Illustrate the Proposed Relationship between Soluble Complexes, Ion Pool, and Solids of Calcium Compounds in Body Fluids

OBSERVATIONS ON THE ROLE OF PHOSPHATASE[182]

WILLIAM F NEUMAN, VICTOR DiSTEFANO, and BETTY JANE MULRYAN

From the Division of Pharmacology and Toxicology Department of Radiation Biology School of Medicine and Dentistry University of Rochester Rochester New York

Armstrong If we can go along now I will ask Dr Neuman to discuss his recent observations on the role of phosphatase

Neuman Phosphatase has been mentioned in almost every scheme of calcification since Robison[183] first suggested its possible importance Perhaps the principal reason for the persistence of this idea is that an almost perfect correlation has been repeatedly observed[184 185 186] between the occurrence of phosphatase histologically and the areas of active calcification

Observations at Variance with Concepts of Role of Phosphatase

Robison himself however reported a number of disturbing observations His original idea was that phosphatase provided local, high concentrations of inorganic phosphate by virtue of its action on suitable ester

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[182] This paper is based on work carried out under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project Rochester New York

[183] Robison R The Possible Significance of Hexosephosphoric Esters in Ossification *Biochem J* **17** 286 293 (1923)

[184] a Robison R and Soames K M The Possible Significance of Hexosephosphoric Esters in Ossification II The Phosphoric Esterase of Ossifying Cartilage *Biochem J* **18** 740 754 (1924)

b Martland M and Robison R The Possible Significance of Hexosephosphoric Esters in Ossification V The Enzyme in the Early Stages of Bone Development *Biochem J* **18** 1354 1357 (1924)

c Fell H B and Robison R The Development and Phosphatase Activity *In Vivo* and *In Vitro* of the Mandibular Skeletal Tissue of the Embryonic Fowl *Biochem J* **24** 1905 1921 (1930)

d Macfarlane M G Patterson L M and Robison R The Phosphatase Activity of Animal Tissues *Biochem J* **28** 720 724 (1934)

[185] Lorch I J Alkaline Phosphatase and the Mechanism of Ossification *J Bone and Joint Surg* **31B** 94 99 (1949)

[186] Gomori G Microtechnical Demonstration of Phosphatase in Tissue Sections *Proc Soc Exp Biol and Med* **42** 23 26 (1939)

of less calcium and less phosphorus as if they were having a precipitate form. Then at longer time period the direction reverses as if calcium were leaving the solution without a change in phosphate concentration.

So it looks to me as if there were at least two things going on. One of them is a change parallel to this solubility line in Figure 52 and the other is something else.

Hastings: But I am sure there is no question about the fact that if you shake a solution that has the ion product of serum with a large amount of either ground up bone or so-called tertiary calcium phosphate, there is very quickly a decrease, and a marked decrease, of the calcium in solution and practically no change in the phosphate.

That is in the literature.

Hodge: Is it?

Hastings: That occurs just as fast as you can shake it up and centrifuge it.

Armstrong: That is the reason it was taking out calcium carbonate below the solubility of calcium carbonate.

Hastings: Yes, but regardless of how it came down, the point is that if you have a solid, if you have a lot of it there, which in our experiments was about 3 gm. per 50 cc. (in those early experiments), you take it out very rapidly, and it does not go out as a calcium phosphate compound. I am sure of that much.

McLean: You are doing the same thing that you were doing with the lead phosphate you spoke of yesterday.

Hastings: That is right, except that it is not as good an adsorber, or whatever you want to call it—calcium remover.

OBSERVATIONS ON THE ROLE OF PHOSPHATASE[*]

WILLIAM F. NEUMAN, VICTOR DISTEFANO, and BETTY JANE MULRYAN

From the Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

Armstrong: If we can go along now I will ask Dr. Neuman to discuss his recent observations on the role of phosphatase.

Neuman: Phosphatase has been mentioned in almost every scheme of calcification since Robison[1] first suggested its possible importance. Perhaps the principal reason for the persistence of this idea is that an almost perfect correlation has been repeatedly observed[2,3,4] between the occurrence of phosphatase histologically and the areas of active calcification.

Observations at Variance with Concepts of Role of Phosphatase

Robison himself, however, reported a number of disturbing observations. His original idea was that phosphatase provided local high concentrations of inorganic phosphate by virtue of its action on suitable ester

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* This paper is based on work carried out under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

(1) Robison, R.: The Possible Significance of Hexosephosphoric Esters in Ossification. *Biochem. J.* 17: 286-293 (1923).

(2)a Robison, R., and Soames, K. M.: The Possible Significance of Hexosephosphoric Esters in Ossification. II. The Phosphoric Esterase of Ossifying Cartilage. *Biochem. J.* 18: 740-754 (1924).

b Martland, M., and Robison, R.: The Possible Significance of Hexosephosphoric Esters in Ossification. V. The Enzyme in the Early Stages of Bone Development. *Biochem. J.* 18: 1354-1357 (1924).

c Fell, H. B., and Robison, R.: The Development and Phosphatase Activity *In Vivo* and *In Vitro* of the Mandibular Skeletal Tissue of the Embryonic Fowl. *Biochem. J.* 24: 1905-1921 (1930).

d Macfarlane, M. G., Patterson, L. M., and Robison, R.: The Phosphatase Activity of Animal Tissues. *Biochem. J.* 28: 720-724 (1934).

(3) Lorch, I. J.: Alkaline Phosphatase and the Mechanism of Ossification. *J. Bone and Joint Surg.* 31B: 94-99 (1949).

(4) Gomori, G.: Microtechnical Demonstration ...
Soc. Exp. Biol. and ...

substrate He found, however, that ester phosphate was present only in low concentrations in plasma[187] Further, phosphatase was relatively ineffectual in hydrolizing these particular esters Following these observations he relegated to phosphatase a role of secondary importance and suggested the existence of a "second mechanism"[188] Though Robison did not clearly distinguish between them, actually, his proposed "second mechanism was comprised of two factors a) an organic matrix which was responsible for the orderly and specific deposition of the bone mineral, and b) a cellular or enzymatic component which was differentiated from the phosphatase mechanism by its sensitivity to a variety of inhibitors

In an attempt to find a means of supplying phosphatase with suitable substrate, recent workers have focused attention on the importance of glycogen and the glycolysis phosphorylative cycle[189 190 191] These workers had been successful in showing the importance of the glycolytic scheme in calcification They have not, however, been successful in demonstrating that the purpose of the glycolytic process is to furnish substrate for phosphatase This may be illustrated by quoting from Gutman,[189] "Whether glycogenolysis is essential in order to provide phosphorus in some special form or concentration or place, or whether required energy is made available, is not clear"

I should like to emphasize this lack of clarity by raising the following question *How can the cell secrete significant quantities of ester phosphate to the surrounding medium without simultaneously removing an equal molar quantity of inorganic phosphate?* This could be possible only if the

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187a Kay H D and Robison R The Possible Significance of Hexosephosphoric Esters in Ossification III The Action of the Bone Enzyme on the Organic Phosphorus Compounds in Blood *Biochem J* 18 755 764 (1924)

b Martland M Hansman F S and Robison, R The Phosphoric Esterase of Blood *Biochem J* 18 1152 1160 (1924)

c Goodwin H W and Robison R The Possible Significance of Hexosephosphoric Esters in Ossification IV The Phosphoric Esters of the Blood *Biochem J* 18 1161 1162 (1924)

188 Robison R Maclead M and Rosenheim A H The Possible Significance of Hexosephosphoric Esters in Ossification IX Calcification *In Vitro Biochem J* 24 1927 1941 (1949)

189 Gutman A B and Yu T F Further Studies of the Relation Between Glycogenolysis and Calcification in Cartilage *Trans Macy Conference on Metabolic Interrelations* 1 11 26 (1949)

190 Follis R H Jr Glycogen in Rachitic Cartilage and Its Relation to Healing *Proc Soc Exp Biol and Med* 71 441-443 (1949)

191 Hoffmann A Lehmann G and Wertheimer F Der Glykogenbestand des Knorpels und seine Bedeutung *Pflugers Archiv fur die gesamte Physiologie* 220 183 193 (1928)

cell involved possesses the ability to store phosphate in a relatively non ionic form Admittedly, recent evidence indicates that such a phenomenon may occur Rothstein and coworkers[19] have presented data which strongly suggest that the yeast cell, at least may contain phosphorus in the form of a highly polymerized meta phosphate For the present however similar data on hypertrophic cartilage cells and osteoblasts are totally lacking

At the moment the crux of the phosphatase question appears to involve two conflicting pieces of information a) the enzyme is invariably found to be present in areas of active calcification suggesting a key role in the deposition of osseous mineral and b) it has been impossible to obtain adequate evidence that the enzyme though present can accomplish much because of a lack of substrate

Inhibitory Effect of Ester Phosphate on Calcification

During the course of the discussions at last year s Conference it occurred to me that ester phosphate may be inhibitory to calcification This proposition presents the phosphatase problem in a new light Provided ester phosphate is sufficiently inhibitory a high concentration of substrate is not required for phosphatase to play an important role in calcification The enzyme may be acting as a trigger mechanism by removing an agent which prevents the occurrence of a general precipitation of calcium salts

A series of experiments were performed to test this hypothesis The first experiments undertaken were designed only to show gross effects We had demonstrated previously[193] that powdered bone preparations are capable of crystal growth when immersed daily in a synthetic ultrafiltrate of plasma This phenomenon was observed again except that in one series a representative phosphate ester was added to the ultrafiltrate The results are given in Figure 57 Clearly the presence of ester inhibited the formation of crystals

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[19] a Rothstein A *et al* The Relation hip of the Cell Surface to Metabol sm IV VI VII University of Rochester Atom c Energy Project Nos UR-69 UR 98 and UR 120

b Rothstein A and Larrabee C The Cell Surface of Yeast as the Site of Inhib tion of Glucose Metabolism by Uranium—UR 8

c Rothstein A Frenkel A and Larrabee C Certain Characteristics of the Complex of Uran um with Cell Surface Groups of Yeast the Relationsh p of These Groups to Glucose Metabolism—UR 17

d Rothstein A The Metabolic Effects of Uranium on the Yeast Cell—UR 54

e Rothstein A Meier R, Hurwitz L Berke H and Larrabee D The Mechanism of Action of Uran um on Cells—UR-80

[193] Neuman W F Bone as a Problem in Surface Chemistry *Trans Macy Conference on Metabolic Interrelations* 2 32 72 (1950)

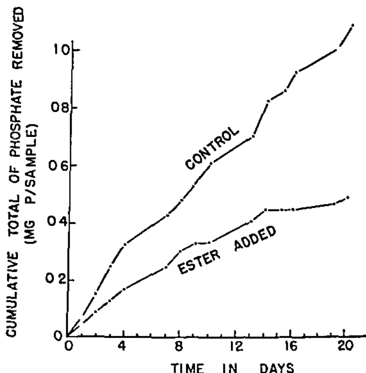


Fig 57 The Inhibition of Mineral Accretion *In Vitro* by the Presence of Glycerophosphate

T ... ve 10 mg % P when completely hydro ... fatted veal bone were placed in 10 ml ... e same composition as solution CP (see ... ° C the suspension was centrifuged the solution aspirated for analysis and fresh solution added In the graph are plotted the amounts of inorganic phosphate removed from the solution by the bone preparation

How the presence of ester phosphate accomplished this inhibition was not evident Two reasonable mechanisms may be suggested a) the ester may form relatively undissociated complexes with calcium thereby reducing *the calcium ion activity or b) the ester may be adsorbed on the crystal* surfaces thereby preventing further additions of calcium and phosphate in the correct lattice positions

Formation of Calcium Complexes by Ester Phosphate

To obtain a partial answer the experiments were repeated, this time however the ester was added as the calcium salt In this case any inhibition observed could be due only to surface adsorption These results are summarized in Table XXVII

In most cases in which the calcium salt of the ester was added crystal formation took place indeed at a rate greater than observed in the controls Clearly then complex formation is indicated At least part of the *inhibitory effect of ester phosphate is due to its ability to form undissociated* complexes of calcium

As a more direct test of this action the following experiment was designed Varying amounts of calcium chloride were added to solutions of sodium sulfate In all instances the final volume temperature and pH were identical and constant In another series varying amounts of calcium chloride were added to solutions containing sodium sulfate and sodium

TABLE XXVII

Effect of Glycerophosphate on Average Daily Crystal Growth

Constituent Added	Fresh Bone		Ashed Bone		Constituent Analyzed
	Control	Experimental	Control	Experimental	
Free Ester	mg./day 0 65	mg /day 0 22	mg /day 0 81	mg /day 0 33	Phosphate (P)
Free Ester	0 51	0 20	0 63	0 24	Phosphate (P)
Calcium Salt of Ester	0 72	1 13	1 11	1 60	Calcium (Ca)
Calcium Salt of Ester	0 38	0 41	0 88	0 71	Phosphate (P)

glycerophosphate When, as in Figure 58, the weight of precipitate obtained was plotted versus the amount of calcium added, the complexing action of ester phosphate was clearly apparent Surprisingly, one mole of ester phosphate was found to bind two moles of calcium The only explanation we have for this unexpected combining ratio is that calcium must interact with hydroxyl groups of the glycerol part of the molecule

Prevention of Crystal Formation by Ester Phosphate

As a more sensitive and direct test of the inhibitory action of ester phosphate experiments were undertaken employing the calcification of rachitic bone slices Like other workers in this field we have evolved our own set of conditions for utilizing this technique Four slices were obtained from each individual rat and one slice placed in each of the following four solutions a) the basic solution (containing inorganic calcium and phosphate) to which nothing had been added, b) a solution containing the basic solution to which calcium glycerophosphate had been added c) a solution containing the basic solution to which mercuric chloride had been added and d) a solution containing the basic solution to which both calcium glycerophosphate and mercuric chloride had been added Preliminary experimentation demonstrated that phosphatase activity as determined colormetrically [94] was consistently inhibited by the concentration of mercury employed Occasional slices did show some residual activity

The results of a series of such experiments are presented in Tables XXVIII XXIX XXX XXXI In those experiments in which phosphatase was not inhibited the addition of ester resulted in increased calcification This effect was neither consistent nor striking In those experiments in which phosphatase was rendered inactive by the presence of mercury the addition of ester inhibited calcification almost without exception It should be emphasized that the inhibitory effect of ester in the calcification experiments cannot be attributed to its ability to complex calcium Actually there was an error in our calculations resulting in the addition of four moles of calcium per mole of ester phosphate in all cases where ester was present in the calcifying solution It should also be emphasized that the concentration of ester phosphate in these experiments was very low approximately 0 125 mg ester P %

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[94]Huggins C and Taly P Sodium Phenolphthalein Phosphate as a Substrate for Phosphatase Tests *J Biol Chem* 159 399–410 (1945)

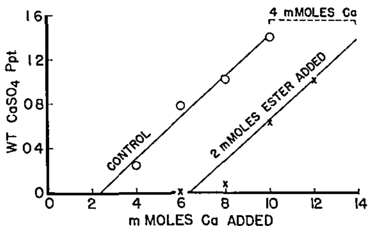


Fig 58 The Complexing of Calcium by Glycerophosphate as Indicated by the Inhibition of the Precipitation of Calcium Sulfate

TABLE XXVIII

The Inhibition of Calcification by Ester Phosphate When Phosphatase Is Inactivated *

CP	CPE	CPI	CPEI	
2½ (++++)	3 (++++)	1 (++++)	1 (++)	37°
2 (++++)	2½ (++++)	1 (+)	0	
2 (++++)	2½ (++++)	1½ (++++)	1 (++)	
1½ (++++)	2 (++++)	1 (+)	0	1°
1½ (++++)	2 (++++)	1 (+)	0	
1½ (++++)	2 (++++)	1 (+)	0	

*Explanation of symbols C=ionic calcium P=inorganic phosphate
E=calcium glycerophosphate I=mercury
The calcification of slices was graded on a scale of 0 4 indicating vertical calcification and 0 4+ indicating transverse calcification when the slice is viewed in its normal position

TABLE XXIX

The Inhibition of Calcification by Ester Phosphate When Phosphatase Is Inactivated *

CP	CPE	CPI	CPEI	
2 (++++)	3 (++++)	1 (++)	0	37°
2 (++++)	2½ (++++)	2 (++)	3 (++++)	
2 (++++)	2 (++++)	1 (+++)	1 (+++)	
1½ (++++)	2 (++++)	1 (+)	0	1°
2 (++++)	2 (++++)	1 (++)	1	
1½ (++++)	2 (++++)	1 (++)	0	

*Explanation of symbols C=ionic calcium P=inorganic phosphate
E=calcium glycerophosphate I=mercury
The calcification of slices was graded on a scale of 0-4 indicating vertical calcification and 0 4+ indicating transverse calcification when the slice is viewed in its normal position.

TABLE XXX

The Inhibition of Calcification by Ester Phosphate When Phosphatase Is Inactivated *

CP	CPE	CPI	CPEI	
3 (++++)	3½ (++++)	2 (++++)	1 (++++)	37°
3 (++++)	4 (++++)	2 (++++)	1 (++++)	
3 (++++)	3 (++++)	2 (++++)	1 (++++)	
2 (++++)	2 (++++)	1 (+)	0	1°
2 (++++)	2 (++++)	1 (++++)	0	
2 (++++)	2 (++++)	1 (+++)	1 (+++)	

*Explanation of symbols C=ionic calcium P=inorganic phosphate
E=calcium glycerophosphate I=mercury
The calcification of slices was graded on a scale of 0 4 indicating vertical calcification and 0 - 4+ indicating transverse calcification when the slice is viewed in its normal position.

TABLE XXXI

The Inhibition of Calcification by Ester Phosphate When Phosphatase Is Inactivated *

CP	CPE	CPI	CPEI	
2½ (++++)	2½ (++++)	1½ (++++)	1 (+)	37°
2 (++++)	2 (++++)	1 (++)	0	
2 (++++)	2 (++++)	1 (+++)	1 (+)	
2½ (++++)	2½ (++++)	0	0	1°
2 (++++)	2½ (++++)	1 (+)	0	
2½ (++++)	2½ (++++)	1 (+)	0	

*Explanation of symbols C=ionic calcium P=inorganic phosphate
E=calcium glycerophosphate I=mercury
The calcification of slices was graded on a scale of 0 4 indicating vertical calcification and 0 4+ indicating transverse calcification when the slice is viewed in its normal position.

Studies with Radioactive Glycerophosphate

These results indicated that ester phosphate must inhibit calcification by preventing crystal formation Such an action could take place only if the ester is actually adsorbed by the mineral substance Accordingly, radio

active glycerophosphate was prepared[195] and carefully purified to insure the absence of contaminating inorganic radiophosphate Solutions containing varying amounts of this ester at physiological pH were shaken with specimens of powdered glycol ashed veal bone for 29 hours Preliminary experimentation had established two facts a) the equilibrium was reached in this period of time and b) the radioactive phosphate present in the ester did not undergo exchange with inorganic phosphate in solution The bone specimens were separated from the solutions and the amount of adsorbed ester determined from the amount of radioactivity taken up by the bone These results are presented in Figure 59

The results could satisfactorily be described by a Langmuir isotherm and indicated that at physiological concentrations of ester, considerable quantities are adsorbed by the bone mineral This finding offers support for the idea that ester phosphate inhibits crystal formation by occupying surface lattice positions

Discussion

With the data reported above it appears that phosphatase has a threefold action in favoring calcification a) it increases the concentration of inorganic phosphate as first suggested by Robison b) it increases the concentration of calcium ion by removing the presence of a complexing agent and c) it prevents the adsorption of ester phosphate and thus eliminates a process which is inhibitory to the growth of the mineral crystals It is difficult to evaluate in a quantitative sense the relative importance of each of these three actions It seems clear however, that phosphatase plays an important role in calcification even though the absolute total concentration of its various substrates may be quite low

Conference Discussion

Gutman Which ester did you use?

Neuman This is *a* glycerol phosphate All the work has been done with *a* glycerol phosphate That is the big weakness in our experiments Time did not permit a fuller exploration

Hastings What was the molar concentration?

Neuman It was 10 mg per cent phosphorus That is equivalent to about 0 003 molar

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[195] King H and Pyman F L The Constitution of the Glycerylphosphates The Synthesis of *a* and *β* Glycerylphosphates *J Chem Soc* 105 1238 1259 (1914)

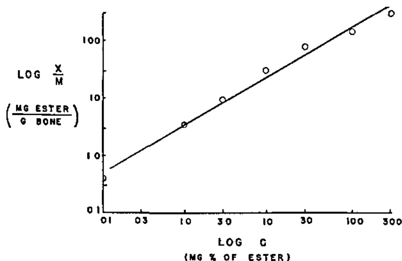


Fig 59 The Adsorption of Radioactive Glycerophosphate by Glycol Ashed Bone

active glycerophosphate was prepared[195] and carefully purified to insure the absence of contaminating inorganic radiophosphate Solutions containing varying amounts of this ester at physiological pH were shaken with specimens of powdered glycol ashed veal bone for 29 hours Preliminary experimentation had established two facts a) the equilibrium was reached in this period of time, and b) the radioactive phosphate present in the ester did not undergo exchange with inorganic phosphate in solution The bone specimens were separated from the solutions and the amount of adsorbed ester determined from the amount of radioactivity taken up by *the bone These results* are presented in Figure 59

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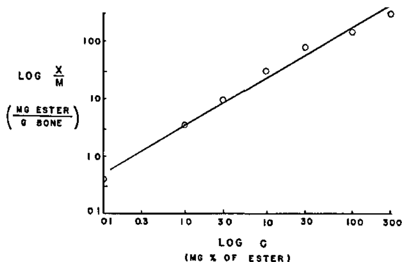


Fig 59 The Adsorption of Radioactive Glycerophosphate by Glycol Ashed Bone

Hastings Is that a lot more ester this time?

Neuman No I will give the actual ratios We added 2 millimoles of ester per 100 cc and the amount of calcium complex as measured by the displacement turned out to be 4 millimoles This was done in an attempt to get a quantitative combining ratio We could not use the polarograph or any of the commonly used techniques in chelate studies But this was our escape

Hastings That would account for your other experimental result wouldn t it?

Neuman Yes As a matter of fact it would go a long way toward explaining it No on second thought that is the wrong direction It is altogether wrong But it is such a crude test It was merely an exploratory experiment and I presented it only because it was our point of departure

Armstrong Did you say that the radiophosphorus of the ester became distributed between the phosphorus of the ester in the mineral phase?

Neuman No it was taken up by the bone If you add inorganic phosphate to a solution of ester and separate the ester the specific activity of the ester is unchanged just as you would expect—particularly with glycerol phosphate Glycerol phosphate is very stable to hydrolysis even at conditions of very high temperature

Gutman When you refer to physiological concentrations of ester do you mean those in the serum?

Neuman Yes

Gutman The concentration in the cell may vary enormously

Neuman I am considering now only the extracellular phase

Gutman And presumably intercellular fluid?

Neuman Yes presumably That is a presumption

Shorr I wonder whether Dr Hiatt would say something about some experiments he did in which β glycerol phosphate was added to a basal solution containing inorganic phosphate and what effect it had upon calcification

Hiatt Using L-histidine 1×10^{-2} M or beryllium 1×10^{-4} M we could demonstrate no inhibition when the medium contained both inorganic phosphorus (5 mg %) and ester phosphate (β glycerophosphate in an amount calculated to yield 10 mg % inorganic phosphorus on complete hydrolysis)

Neuman In the presence of the ester?

Hiatt We could detect no inhibition of calcification by beryllium 1 × 10^{-4} M, in the presence of inorganic phosphate whether or not ester phosphate was present in addition

Neuman Alone?

Hiatt That is right

Neuman That would seem contrary, I would say On the other hand for your data, Dr Gutman you did not have inorganic phosphorus present

Gutman In most experiments only phosphoric ester was employed but in some both ester and inorganic phosphate were present In the latter experiments no inhibition of calcification was observed as Dr Hiatt found

Neuman Waldman's data fit in very well with this view He demonstrated a lack of calcification in the presence of ester when phosphatase was inhibited by heat

This is very tricky, and I should like very much to do this over with beryllium because it seems to me beryllium is a much better inhibitor than mercury might be I will not say that this is an established fact because I feel that the rachitic slice technique is very tricky In the way we set it up it came out right We have demonstrated the reasons why it should come out that way Ester is actually absorbed by the mineral and inhibits crystal formation by a complexing calcium I would be surprised if every time it can be demonstrated that ester does inhibit calcification of rachitic slices

Hastings I wish you had some other esters

Neuman I do too I think that some of the others should be equally good or better They might not

Armstrong Robison and Rosenheim[19] in their studies of *in vitro* calcification tried insulin and, as I recall they could note no effect of insulin which was different from that of any other protein Of course when one puts protein in the calcifying solutions interferences with calcification are produced

Shorr I wonder whether in line with our present thinking we should not take the Robison Rosenheim concept of the second mechanism and put it first The preparation of an ester must come first, and then subsequently it would be broken down The second thing that I was not clear

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[19a] Robison R and Rosenheim A H Calcification of Hypertrophic Cartilage *in Vitro* *Biochem J* 28 684 (1934)

about was your objection to phosphate going into the cartilage cell as a primary part of the building up of glycogen Why did you feel that you could not accept that as one of the mechanisms operating? How does the glycogen get into the cell?

Neuman This gets into a pretty rugged field but, as I understand it now, from the gist of the opinion of people like Kamen and Spiegelman,[197] Sacks,[198] and Rothstein [199] phosphate enters the cell only accompanied by glucose, and probably in an equal molar quantity However, glycogen does contain phosphate but not in equal molar quantity with glucose Thus, if I try to bring in sufficient phosphate to permit all of this glycogen to be emitted again, or secreted as an ester, I have put in a tremendous amount of inorganic material which would, I think, disturb the electrolytic balance within the cell With regard to the question of how the phosphate gets out again obviously the cell metabolized glycogen to CO_2, and if every molecule of glucose comes in taking phosphate with it, why do not the cells not fill up with phosphate? So there is something very wrong with the over all idea Would you care to comment on that?

Hastings No I would agree that the glucose and phosphate go into it mole for mole

Follis Could that be the thing that kills the cell, because you are right at the point where the cell is either dying or killed by the blood vessel

Neuman Possibly I am not denying the fact that the cell may furnish substrate I want that clear There may be a local mechanism, a cellular mechanism, for providing large quantities of ester The point I am trying to make is that we do not have to discard phosphatase as an important factor in calcification because we have not, as yet, demonstrated a large quantity of substrate on which it might act In these experiments, a very small quantity of ester consistently acts as an inhibiting influence and, therefore, phosphatase would act in a beneficial way, even though the sub strate concentration is low

It also would explain, perhaps in part, how pathological calcification seems to correlate best with those tissues which also have phosphatase activity

Armstrong Since we are indulging in speculation I can remind you of a paper by Williams [200] that appeared in *Nature* about a year and a half

[197] Kamen M D and Spiegelman S Studies on the Phosphate Metabolism of Some Unicellular Organisms *Symposia on Quantitative Biology*, Cold Spring Harbor XIII 151 163 (1948)

[198] Sacks J Mechanism of Phosphate Transfer Across Cell Membranes *Ibid* p 180-184 (1948)

[199] Rothstein A Discussions *Ibid* p 162 and 184 (1948)

[200] Williams W T Function of Urease in Citrullus Seeds *Nature* 165 79 (1950)

ago The author suggested the idea that we may be able to demonstrate an *in vitro* activity of a protein as an enzyme which may have no relation ship to the physiological function of the protein He used as the experi mental basis for this theory urease of watermelon seed He found on sprouting the seeds that the urease content decreased rapidly The idea proposed is that the urease in the seeds has nothing to do with hydrolyzing urea It represents a form by which the seed stores protein He suggests that it is merely accidental that this particular protein hydrolyzed urease

If you carry this idea further you have to recognize the possibility that it may be that phosphatase hydrolyzes phosphoric acid esters *in vitro* with out any relation of this type of hydrolysis to bone formation We are struck however with the point that we would like to see some utilization of this hydrolytic power of the enzyme in bone formation

Neuman I think we are all more logical than we care to admit

Hastings I would not agree with you If you have a glycolytic cycle of any kind you are going to have phosphorus around too It is part of the system I am not talking about a specific effect

Shorr There would seem to be no justification for ruling out the par ticipation of phosphatase merely because the prevailing local concentration of organic phosphate esters is low All that would be required is that there is a continuing supply which would keep up that concentration

A more important consideration is the nature of the organic phosphate ester upon which the bone phosphatase would act If it could act on all the phosphate esters in the phosphorylative glycolytic cycle then there would seem to be little reason for the cycle proceeding through all of its steps The essence of the problem might lie in the accessibility of the intra cellular phosphate esters to the phosphatase which is extracellular Thus although phosphatase may split any organic phosphate ester in the glycolytic series it may have access to one specific ester which the cartilage cell releases extracellularly

Gutman I have one possible explanation for that difficulty Dr Shorr I think it may depend upon differences in the rates of action of the various enzymes involved

Alkaline phosphatase as Dr Shorr has indicated is nonspecific in its action and will dephosphorylate a great many phosphoric esters including glucose 1 phosphate glucose 6-phosphate fructose diphosphate and other phosphoric esters in the glycolytic cycle but will do so at a very slow rate whereas the action of phosphorylase in converting glycogen to glucose-1 phosphate and that of phosphoglucomutase and all the other enzymes in the glycogenolytic cycle is extremely rapid Consider for example, the

position of glucose 1 phosphate which is subject to the action of three separate enzymes namely phosphorylase which may convert the glucose 1 phosphate back to glycogen phosphatase which may dephosphorylate to glucose and phosphoglucomutase which may convert glucose 1 phosphate to glucose 6-phosphate When glycogenolysis takes place the point of equilibrium is such that the major part of glucose 1 phosphate is converted to glucose 6-phosphate So I think the crux of the paradox lies in different rates of activity of the various enzymes concerned

Shorr Would you not have to consider not only rate but concentration?

Gutman And the concentrations of enzyme and substrate which determine in large part the direction and equilibrium point of the reaction

Shorr I am just thinking of the fact that we are working on rachitic rat slices Actually perhaps we ought to go to the growing deer antler just as Aub and Wislocki did because the concentration of alkaline phosphatase at the growing points is very great and really astronomical compared with what we are dealing

Neuman After sitting here I have become a little more oriented and I would like to ask one more question because these contrary data are rather unhandy to say the least

We were able to demonstrate that with 1000th molar that is 10^{-3} mercury we did not get consistent inhibition of phosphatase The probability of removal of metallic enzyme inhibitors by the mineral presents the possibility that you also did not have phosphatase inhibited in all cases I wonder if you demonstrated the inhibition of phosphatase by colorimetry or some other means at 10^{-4} Be^{++}

Hiatt We are not sure that the effect on calcification was the result of inhibition of alkaline phosphatase We did notice that in the presence of phosphate ester without inorganic phosphate there was no calcification

Neuman And you noticed in the presence of inorganic phosphate the ester did not act as an inhibitor But the question is Was the phosphatase inhibited in that experiment?

Hiatt Yes we agree that the question remains to be answered

Neuman Did you demonstrate it or do you assume that 10^{-4} Be inhibited it?

Hiatt We did not demonstrate it We also are not sure that inhibition of alkaline phosphatase is the mechanism of interference with calcification in our experiments

Neuman So we may not be in disagreement

Follis I think we should go easy on the statement that phosphatase is nonspecific and that it is based on some of Jonas Friedenwald s histochemical studies, [91] by the use of inhibitors and specific substrates For instance, he finds he can demonstrate one phosphatide in the glomerulus in the kidney and another phosphatide in the tubules So not the same thing may hold true

Gutman Your objection is well taken But using bone or cartilage phosphatase one can demonstrate dephosphorylation of β glycerophosphate α glycerophosphate phenylphosphate glucose 1 phosphate glucose 6 phosphate fructose diphosphate ATP and many other phosphoric esters

Follis But there may be a number of dephosphorylating enzymes present

Gutman Yes that is perfectly true

Neuman That may be Robison himself I believe got all worried because he found that the phosphate preparations he started with did not hydrolyze blood esters Then he made a preparation of bone phosphatase which did act on blood esters to his obvious relief

Sobel I should just like to add for whatever confusion it may bring that Professor Albaum of Brooklyn College working in connection with our group measured the ATP content of rachitic bone and found it to be 0 1 as rich as muscle with respect to ATP This represents a very rich source of organic phosphate These experiments have been repeatedly confirmed

Gutman Has this work been published?

Sobel No it is just preliminary just started

Hastings Any ACTH?

Sobel He used it for measuring the distribution of ATP and ADP It is just the ATP angle that concerns him His preliminary results which were confirmed indicate this bone is high in ATP

Follis Periosteum will hydrolyze

Gutman We looked for adenosine triphosphatase in cartilage and found minimal activity as measured by the method of Dubois and Potter

Armstrong Dr Bevelander do you have observations from your studies of invertebrates that would bear on this discussion?

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[91] Moeng yn Davies G D and Friedenwald J S Histochemical Studies on the Alkaline Phosphatases in the Tissues of the Rat Using Frozen Sections *J Cell and Comp Physiol* 36 421 (1950)

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Bevelander You mean to add more confusion to the situation (Laughter)

In regard to phosphatase some time ago we made a study of the relation of phosphatase in developing teeth particularly in reference to histogenesis of dentin and enamel and came to about the same kind of conclusions that have been reached in reference to the correlation between the presence of phosphatase in the development of bone We were not quite satisfied with these results and from there we went on to some other dermal derivatives such as hair feathers nails and a few other structures We found about the same kind of correlation between the presence of phosphatase in the developing organs that one can observe in enamel dentin and bone

A few years ago I had the opportunity to go to Bermuda and do what I thought was to be a study of the bone of some deep-sea animals I was trying to avoid some laboratory work (Laughter) Several things happened and instead of working on deep-sea animals I worked on a number of mollusks I thought perhaps that some of the invertebrates might have a simpler method of calcification than that which occurs in the higher vertebrates

We started working on a number of different mollusks and during the course of some exploratory experiments we did a number of studies in regard to the way in which crystals were deposited We made some exploratory studies on a membrane which this animal elaborates which looks very much like osteogenic fibers although it does not have the same chemical composition Among other studies that we made was a series of histochemical studies and I was particularly interested to see whether phosphatase was present in that part of the mollusk which is generally accepted to be intimately concerned with laying down or the deposition of the shell

The process of laying down the shell consists of the elaboration of a thin sheet or strip of a fibrous like material which is known as periostrachum or concholin which has a fairly definite chemical composition in terms of its amino acid sugar analysis but which is not comparable to collagen Shell formation can be separated into two processes the first consists of the elaboration of a delicate protein membrane, the second the impregnation of this membrane by very minute crystals which have been observed to undergo considerable growth in our study preparations

But to get back to the phosphatase picture in these animals we do find a very intense phosphatase localization in this portion of the mantle tissue The interesting part about this exoskeleton if you want to call it that is that it is made up of calcium carbonate In the various mollusks this is either aragonite or calcite

In regard to the presence of phosphatase it is a little difficult to see how phosphatase would be utilized in splitting off phosphate ions to produce a calcium carbonate crystal Some of the other possible functions which this enzyme might perform is suggested by the work of Dugal[202] who found when these animals were removed from their aquatic environment that an etching of the inner surface of the shell takes place, which he suggests is correlated with a glycogenolytic process This enzyme has also been de scribed in the spinning gland in the silk worm by Bradfield[203] In this connection this latter investigator suggests that phosphatase may be con cerned with the elaboration of protein fibers

Shorr What about the cartilaginous tissues?

Bevelander Dr Lorch has made a study of that problem Dr Shorr and she finds that there is a very definite localization of alkaline phospha tase during the development of the cartilaginous tissues Dr Lorch worked on older material than the material I worked on up to and including those stages in which the cartilaginous tissue calcifies I want to qualify that calcification It is not the usual bone calcification It consists of an accre tion of calcium crystals around the cartilaginous skeleton Under those conditions she did find the phosphatase

Shorr But you also found it earlier

Bevelander I found it in very very young dogfish embryos

Hendricks Could it be that the function of alkaline phosphatase is in volved with the introduction of serines in proteins since they possibly go through a phosphorylating series?

Bevelander There was an idea that we had with regard to the possible function in connection with calcification There is a possibility that that phosphatase may phosphorylate some substance in that membrane, some thing we do not know about One of the things we had in mind was pos sibly an excess organic calcium salt

Hastings I think there is no doubt now but that high energy phosphates are essential for the formation of the peptide bond Dr Bevelander s demonstration caps the climax that you get lots of phosphatase where you are not making any calcium phosphate salt at all Therefore I think we should direct our attention completely away from the business of the relation between phosphatase as an inorganic phosphate producer

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[202] Dugal L P The Use of Calcareous Shell to Buffer the Product of Anerobic Glycolysis in Venus Mercernaria *J Cell and Comp Physiol* **13** 235 251 (1939)

[203] Bradfield J R G Phosphatase Cytochemistry in Relation to Protein Secretion *Exper Cell Res (Supp I)* p 338 349 (1949)

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Lindstrom I am not willing to throw that notion away entirely It may be that nature is very economical and that she has arranged for an enzyme to do two things It seems to me that we ought to retain in the back of our minds the hydrolytic function of enzymes until we can entirely discount this role What the end of this story will be I have no way of knowing

Neuman I would like to point out that part of our demonstration of ability of ester phosphate to inhibit calcification involved the precipitation of calcium sulfate Perhaps I should say there is a weakness there that I did not admit When we mixed calcium and the solution of sulphate containing ester no precipitation took place even at high concentrations of calcium It was three weeks before we obtained maximal precipitation We have not established that equilibrium is complete in three weeks I believe it is There was no further visible accretion in the last couple of weeks For some reason in the presence of a tenfold excess of calcium away over the solubility product no precipitation took place One might say that possibly the ester phosphate in the fluids of the mollusk—presumably they use ester phosphates in their metabolic schemes too—could be there to prevent the precipitation of calcium carbonate At least that is a possibility

Hastings I am sure that that delay of precipitation is a nonspecific reaction Everyone of us who played with making precipitates of calcium salts from supersaturated solution finds the most amazing high effect of *small quantities of other things present You can have a supersaturated* solution of calcium carbonate and very supersaturated that will precipitate and come to equilibrium very quickly if you only have sodium chloride and sodium bicarbonate and calcium there but a tenth of a millimole per liter of citrate will make the calcium carbonate stay up indefinitely except that I have not kept the solution that long

You can make a solution that is supersaturated with respect to calcium carbonate and put in one millimole of phosphate or one half millimole of phosphate (not to exceed the solubility product of tertiary calcium phosphate) it will stay supersaturated unless you shake it with solid phosphate These supersaturation effects are tremendous

Hendricks I should like to comment as a crystallographer on those particular matters The Shell Development Company in California brought ammonia into use for introduction into irrigation waters They got along all right for about a week After that calcium carbonate began to precipitate and stopped the distribution systems Naturally the question arose as how to solve the difficulty The chemist assigned to the problem tried everything he had in the laboratory and soon found that Calgon sodium hexametaphosphate worked quite well He added a boxful to one irrigation ditch but the company was economically minded and set another man to

work on the problem He started with the idea that the amount of Calgon would depend upon the sequestering of the calcium Instead it soon appeared that very little hexometaphosphate was required Obviously the mechanism had very little to do with the sequestering of the calcium

You can take a calcium bicarbonate system add sodium hexametaphosphate to it and the calcium carbonate will not precipitate Upon standing a very long time it will precipitate but then the crystals have very unusual forms occurring as war club like crystals which are oftentimes found in plant cells and I believe sometimes in animal cells

The explanation seems to be the following that in order for a crystal to grow it has to grow at a nucleation center It has to grow at a center of some type that is present These nucleation centers as a rule are motes that is any sort of a dust particle Before a crystal can continue to grow it has to have fluctuated beyond a certain size because if it is less than a certain size apparently it will redissolve go right back in The function of these additive materials is to poison the mote as a nucleation center for the growth of the crystal

I do not know I suspect that that has nothing to do with the formation of bone but it may have a great deal to do in the mollusks with the prevention of deposition of calcium carbonate in quite a morphologically wrong position In other words it is a morphological factor that prevention of precipitation in one position and encouraging it in another might be relatively important and those characteristics must play a part in morphogenesis in lower forms

Bevelander As a matter of fact we have seen something like that happen In some of these animals we introduced cover slips and got shell crystal growth to take place on an ordinary micro cover slip We saw crystals of all sizes and all variations and a great many aberrations in crystal pattern

Copp What is the explanation of the very high phosphatase in rachitic animals?

Sobel You mean in the blood?

Copp In the blood and in the bone

Neuman I have no idea

Follis There is increased osteoblastic activity and the phosphatase is usually taken to indicate if there is no liver disease increased osteoblastic activity

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THE EFFECT OF AGE AND LOW PHOSPHORUS RICKETS ON CALCIFICATION AND THE DEPOSITION OF CERTAIN RADIOACTIVE METALS IN BONE [204]

D H COPP [205], J G HAMILTON[206], D C JONES [207], D M THOMPSON [208] and C CRAMER [209]

From the Department of Physiology, Faculty of Medicine University of British Columbia Vancouver B C and the Divisions of Physiology Medical Physics Experimental Medicine and Radiology and Crocker Laboratory University of California Berkeley and San Francisco California

Armstrong The topic for this afternoon is Metabolic Turnover of the Skeleton It will be introduced by Dr Copp

Some of you may not know that Dr Copp has recently returned to Canada where he is the Professor of Physiology in the Medical School of the University of British Columbia in Vancouver

Copp Radioactive isotopes have made possible new techniques for study of metabolic turnover in the skeleton In addition many of the radioactive elements involved in nuclear fission are deposited in the skelton where the chronic effects of their radiations may produce bone atrophy or bone tumors The hazard to health quite similar to that from chronic radium poisoning has been emphasized by the growing importance of atomic fission from an industrial and military point of view It was this problem that first led us to investigate the deposition of metals in bone [210]

In metabolic behavior most of these metals appear to fall into one of

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[204]This paper is based on work carried out under Contract No W 7405 eng-48A between the Radiation Laboratory of the University of California and the Manhattan Project and U S Atomic Energy Commission

[205]Professor of Physiology University of British Columbia Vancouver Canada

[206]Professor of Medical Physics Experimental Medicine and Radiology University of California Berkeley and San Francisco California

[207]Present address Bio-Medical Branch U S Naval Radiological Defense Laboratory San Francisco 24 California

[208]Present address Department of Zoology University of Arizona Tucson, Arizona

[209]Present address Department of Physiology University of California Berkeley California

[210]Copp D H Axelrod D J and Hamilton, J G The Deposition of Radioactive Metals in Bone as a Potential Health Hazard *Am J Roentgenol* 58 10 16 (1947)

the following groups [211] (a) Alkaline earths such as calcium strontium barium and radium which are localized almost exclusively in the skeleton and which are generally affected by the same conditions which affect calcium metabolism (b) Metals such as yttrium and plutonium, which show some distribution in soft tissues but which are chiefly localized in bone (c) Metals such as cerium lanthanum americium and curium which are initially taken up by the liver in large amounts as well as by bone These metals disappear from the liver within a few months but remain quite firmly fixed in the skeleton

Effect of Age and Low Phosphorus Rickets on the Retention of Certain Radioactive Metals in Bone

The retention of the metals Ca^{45} $Sr^{89\ 90}$ Ba^{140}, Y^{88} and Ce^{144} in bone was compared in the following groups of animals (a) mature adult rats in which bone growth had practically ceased (b) young growing rats in which there was active growth of new bone and (c) young rats with low phosphorus rickets in which new osteoid matrix was forming but deposition of bone salt did not take place

All the animals used were female rats of the Long Evans strain The adult rats were 6 to 12 months old and showed no appreciable weight gain The young normal animals were weaned at 21 days to the stock diet and were 7 to 10 weeks old when used for these experiments The rachitic rats had been weaned at 21 days and reared on a synthetic diet[21] very low in phosphorus (0 008 to 0 015% P) although adequate in calcium (0 43 to 0 46% Ca) The animals were 7 to 10 weeks of age when used and were x rayed to determine if florid rickets was present

The following radioactive isotopes were used

Ca^{45} —5 microcuries of radiocalcium with 1 25 mg Ca as calcium chloride injected intravenously

$Sr^{89\ 90}$—5 microcuries of carrier free radiostrontium (produced by nuclear fission) injected intraperitoneally

Ba^{140} —5 microcuries of carrier free radiobarium (produced by fission) injected intraperitoneally

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211 Hamilton J G The Metabolic Properties of the Fission Products and the Actinide Elements *Rev Mod Phys* 20 718 7_8 (1948)

21 Coleman R D Becks H Kohl F vanN and Copp D H Skeletal Changes in Severe Phosphorus Deficiency of the Rat *Arch Path* 50 209 232 (1950)

Y^{88} —5 microcuries of carrier free radioyttrium (produced in the cyclotron) as chloride in isotonic saline at pH 4 injected intramuscularly

Ce^{141} —5 microcuries of carrier free radiocerium (produced by fission) as chloride in isotonic saline at pH 4, injected intramuscularly

Following injection of the respective radioactive isotope the animals were placed in individual metabolism cages and urine and feces were collected separately They were sacrificed 8 days later and tissues and excreta were analyzed for radioisotope The results obtained are given in Table XXXII The values given for Ca* Sr* and Ba* are expressed as per cent

TABLE XXXII

Effect of Age and Low Phosphorus Rickets on the Bone Uptake of Ca Sr Ba Y and Ce in the Rat

	Per Cent of the Absorbed Dose			
	Skeleton	Urine	Feces	Liver
Ca^{45} (125 mg Ca)				
Normal—Adult	31 7	4 7	43 2	—
Normal—Young	73 4	12 7	7 2	—
Rachitic—Low P	13 4	74 5	1 7	—
$Sr^{89\ 90}$ (Carrier free)				
Normal—Adult	29 1	42 7	26 9	—
Normal—Young	71 9	16 4	12 8	—
Rachitic—Low P	20 4	69 4	7 5	—
Ba^{140} (Carrier free)				
Normal—Adult	37 3	23 2	31 8	—
Normal—Young	61 8	12 4	25 3	—
Rachitic—Low P	33 9	52 4	11 4	—
Y^{88} (Carrier free)				
Normal—Adult	70 5	18 6	5 2	5 7
Normal—Young	76 8	15 1	4 6	3 5
Rachitic—Low P	74 1	8 4	6 7	6 2
Ce^{141} (Carrier free)				
Normal—Adult	42 6	8 2	5 9	43 5
Normal—Young	61 5	5 7	12 0	21 8
Rachitic—Low P	72 4	4 3	5 0	18 3

of the administered dose, for Y^* and Ce^* they represent the per cent of the isotope adsorbed from the site of intramuscular injection Each figure represents the average of values from 5 to 8 animals

OBSERVATIONS

Ca^{45}

A significant part of the dose of radiocalcium was retained in the skeleton of the adult animals Since very little new bone salt is being formed in these animals this is probably accounted for by ion exchange with calcium already present in the bone In young growing animals the retention is much greater and may represent radiocalcium actually incorporated in the newly formed bone salt A similar effect of age on the retention of P^{32} (as phosphate) in bone has been observed by Falkenheim in mice [13] and by Weissberger and Harris in rats [214] There was very little radiocalcium remaining in the rachitic animals when sacrificed and this may have been due to inability to form new bone salt The radiocalcium was lost primarily in urine as had been observed by Weissberger and Harris[215] when radiostrontium was given to rachitic rats

$Sr^{89\ 90}$

The retention of radiostrontium in bone was very similar to that of Ca^{45} in these three groups of animals However the adult animals excreted almost ten times as much of the $Sr^{89\ 90}$ in urine and this was associated with a smaller proportion in the feces

Ba^{140}

Although qualitatively similar to radiocalcium and radiostrontium there was a greater relative retention of radiobarium in adult and rachitic animals This may be due to the decreased solubility and shift in ion exchange

Y^{88}

Neither age nor rickets appeared to have any significant effect on the retention of radioyttrium in bone suggesting that its metabolism must be quite different from that of the alkaline earths This was confirmed by the difference in the distribution of radioautographs described below

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[13]Falkenheim M The Influence of Growth on the Phosphorus Metabolism of the Mouse and the Effect of Thyroxin at Various Ages *Am J Physiol* 138 175 179 (1942)

[214]Weissberger L H and Harris L S Effect of Tocopherols on Phosphorus Metabolism *J Biol Chem* 151 543 545 (1943)

[215]Weissberger L H and Harris L S A Possible Vitamin D Assay Technique with Radiostrontium *J Biol Chem* 144 287 (1942)

Ce^{141}

Age and rickets also appear to have very little effect on the bone fixation of radiocerium. The smaller proportion in the skeleton of the adult animal is accounted for by the larger amount in the liver. The proportion of the dose in the latter is extremely variable and appears to depend on form and methods of administration.

Radioautographs

Radioautographs were prepared from thin undecalcified sections of bone by the technique of Axelrod.[216] The bone sections and corresponding radioautographs are shown in Figures 60 to 65.

Figure 60 shows the distribution of radiostrontium in the femur from a normal young rat. The isotope is found throughout the bone with particularly heavy deposits in the new bone which is forming below the epiphysis. In Figure 61 the distribution in the femur of a rachitic rat is shown. While considerable radiostrontium is found in the thin mineralized shaft of the bone there is none in the undecalcified osteoid matrix beneath the epiphysis. Radioautographs of bones from animals injected with Ca^{45} are very similar. The radiocalcium is found *only* in areas in which bone salt is present and none is found in the uncalcified osteoid matrix of rachitic bone.

This is in contrast to the behavior of radioyttrium (Figure 62) and radiocerium (Figure 63). These two elements in common with plutonium, zirconium and many other heavy metals[210] show a marked concentration in the uncalcified osteoid matrix of rachitic bone. There appears to be a specific affinity of these metals for bone protein even in the absence of bone salt. This is further evidence that their deposition in bone is not analogous to that of calcium.

Radiocerium has a peculiar spotty distribution throughout the dense cortex of bone which is also evident in the radioautograph of the distribution of americium in adult bone (Figure 64). A high power magnification of the latter (Figure 65) shows the radioactive material in the neighborhood of the small blood vessels which run in the cortex. Although the cause of this perivascular distribution in cortical bone is not known, it is commonly associated with the lanthanide earths and americium and curium which are usually in the trivalent state and have very similar physical and chemical

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[216] Axelrod, D. J. An *Improve*[illegible] r Cutting Undecalcified Bone Sect ons and Its Application [illegible] utogr[illegible] c 98 19 2[illegible] (1947)



Fig 60. Femur from a Young Normal Rat Injected with Radiostrontium and Sacrificed 1 Week Later

Note the $Sr^{89\ 90}$ deposited in the shaft and the calcified areas below the epiphysis [From Hamilton *Rev Mod Phys* 20 718 (1948)]

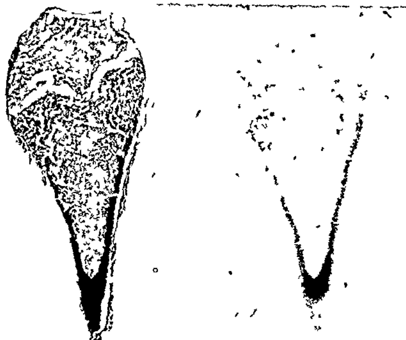


Fig 61 Femur from a 6 Week Old Rat with Low Phosphorus Rickets Injected with Radiostrontium and Sacrificed 1 Week Later

Note that the $Sr^{89, 90}$ is deposited in calcified areas only with none in the uncalcified osteoid matrix below the epiphysis [From Copp Axelrod and Hamilton *Am J Roentgenol* 58 10 (1947)]

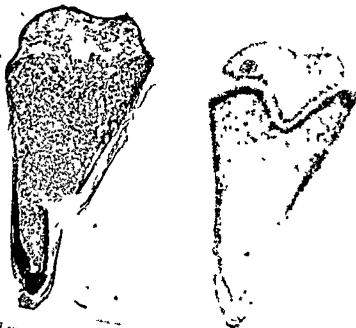


Fig 62 Femur from a 9 Week Old Rat with Low Phosphorus Rickets Injected with Radioyttrium and Sacrificed 1 Week Later

Note the superficial deposition of Y^{88} in the shaft and the heavy deposits of the metal in the uncalcified osteoid matrix below the epiphysis [From Copp Axelrod and Hamilton *Am J Roentgenol* 58 10 (1947)]

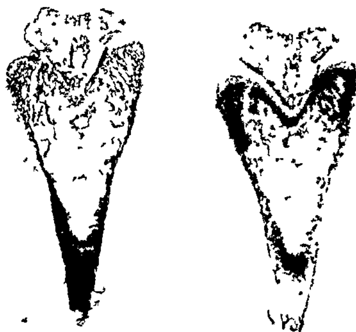


Fig 63 Femur from a 6 Week Old Rat with Low Phosphorus Rickets Injected with Radiocerium and Sacrificed 1 Week Later

heavy depos ts n the
tered po nts of rad o
n lton *A J Roent*



Fig 64 Femur from an Adult Rat Injected with Americium and Sacrificed 16 Days Later

Note the spotty distribution of the Am^{241} within the shaft similar to the distribution of radiocerium [From Hamilton, *Rev Mod Phys* 20 718 1948)]

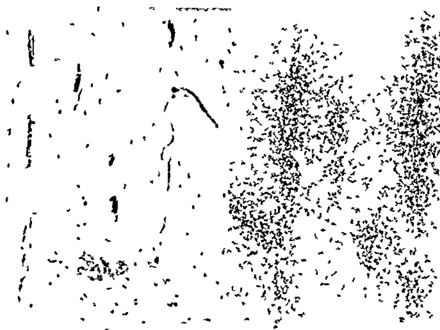


Fig 65 Higher Power Magnification of the Section of Femur and Americium Radioautograph Shown in Figure 64

Note the deposition of americium in the region around the small blood vessels of the cortex of the shaft [From Hamilton *Rev Mod Phys* 20 718 (1948)]

properties These elements are also taken up by the liver in large amounts initially[211]

Effect of Age and Low Phosphorus Rickets on the Turnover of Calcium and Strontium in the Skeleton

The marked effect of age and rickets on the retention of radiocalcium and radiostrontium prompted a more detailed investigation to study the changes responsible for these differences The original work was carried out with radiostrontium[217] but was repeated later using Ca^{45} since the latter is the element normally involved in bone metabolism[18]

Experimental

Isotopes

The radiostrontium used was carrier free and consisted of a mixture of Sr^{89} and Sr^{90} formed by nuclear fission Since Sr^{90} decays to a radioactive daughter Y^{90} with a 60 hour half life all samples were held for 24 days before counting so that equilibrium might be attained and any Y^{90} present at the time of the experiment would have decayed A dose of approximately 5 microcuries of radiostrontium in 0 25 ml neutral isotonic saline was injected intraperitoneally into each animal

The dose of radioactive calcium used contained 5 microcurie of Ca^{45} with 1 25 mg Ca as calcium chloride in 0 25 cc of solution This was administered by intravenous injection The amount of carrier calcium present was found to be sufficient to raise the level of serum calcium as much as 25%, but later experiments with high specific activity calcium (0 01 mg Ca per microcurie) indicated that this amount of carrier had no significant effect on the distribution and excretion of the isotope

Animals

The adult animals were mature female rats of the Long Evans strain from 6 to 12 months old These were raised and maintained on the regular stock Skeletal growth was at a minimum in these animals

The animals with low phosphorus rickets were prepared according to the method described by Coleman *et al*[2,2] These were weaned at 21 days and fed a diet high in calcium (0 43%) and very low in phosphorus (0 03%) for 5 to six weeks They were 9 weeks old and weighed approximately 90

[217]Jones D C and Copp D H The Metabolism of Radioactive Strontium in Young and Rachitic Rats *J Biol Chem* 189 509 514 (1951)

[218]Thompson, D M and Copp D H (Unpublished data)

to 100 grams at the time of the injection of radiocalcium or radiostrontium

The young normal animals were fed a complete diet from weaning until they were 7 to 9 weeks old, at which time they weighed approximately 200 grams They were then injected with the radiocalcium or radiostrontium

Procedure

After injection of the radioactive isotope, the animals were placed in individual metabolism cages, and urine and feces were collected separately The rats were sacrificed at various time intervals from a few minutes to 16 days after the injection In all, 273 animals were used

At autopsy, the femur was removed and analyzed for radiostrontium or radiocalcium, the total amount in the skeleton was estimated according to the method described by Jones and Copp[217] An aliquot of serum was analyzed, and the total serum $Sr^{89,90}$ or Ca^{45} was estimated using the value of 2 4% of body weight reported by Berlin *et al*[219]

The results are shown graphically in Figures 66 to 71 Each point represents the average of values from 4 to 8 animals

A Effect on the Metabolism of Radiocalcium

Serum

Figure 66 shows the amount of radiocalcium present in the serum during the first 4 hours following intravenous injection The fall is rapid in all three groups, but is relatively slower in the adult animals, and remains at a consistently higher level This reflects the slower removal by skeleton and kidney in the adults

Skeleton

The radiocalcium present in the skeleton during the first four hours is shown in Figure 67 and for the first 16 days is shown in Figure 68 It is apparent that the initial uptake by the bones of both normal and rachitic young animals is very rapid However, while the isotope appears to remain fixed in the skeleton of the normal animals, it is rapidly lost from the bones of the animals with low phosphorus rickets This agrees with the findings with radiostrontium, and suggests a very labile calcium fraction in bone which rapidly reaches equilibrium with the serum radiocalcium, and declines as the isotope is excreted in the urine and the serum level falls The ratio of Ca^{45} in bone to that in serum indicates that some 15% of the bone calcium is in the "labile" fraction The significance of this fraction in the calcification mechanism merits further investigation In the adult

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219 Berlin N I Huff R L Van Dyke D C and Hennessy T G The Blood Volume of the Adult Rat as Determined by Fe^{59} and P^{32} Labelled Red Cells *Proc Soc Exp Biol and Med* 71 176 178 (1948)

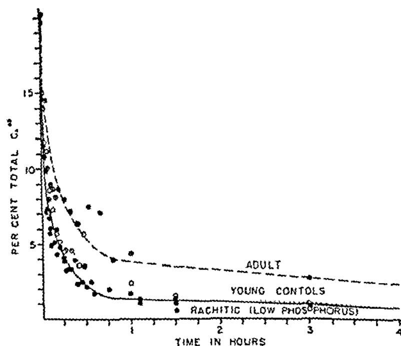


Fig 66 Per cent of the Administered Dose of Radiocalcium in Serum following Intravenous Injection

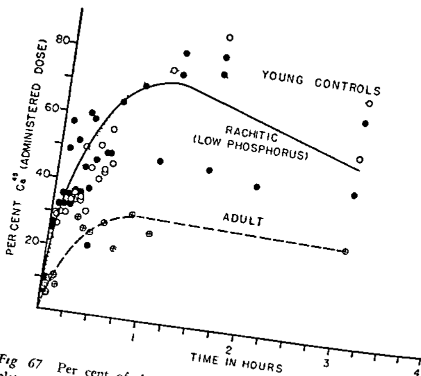


Fig 67 Per cent of the Administered Dose of Radiocalcium in the Skeleton (Estimated) following Intravenous Injection

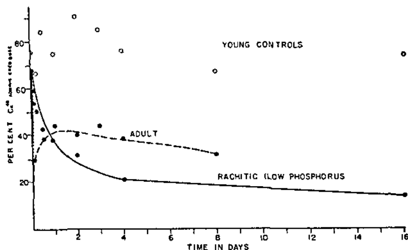


Fig 68 Per cent of the Administered Dose of Radiocalcium in the Skeleton following Intravenous Injection

animals the uptake by the skeleton is much slower reaching a maximum at 2 to 4 hours as has been observed by Norris *et al*[20] It then declines slowly The uptake probably reflects ion exchange with the calcium already present in bone salt since there is little or no new bone formation in these animals Less than ¼% of the bone calcium appears to be involved in this reaction

Urine

The urinary excretion of radiocalcium was similar in both young and adult normal animals (see Figure 69) but was tremendously increased in the rats with low phosphorus rickets Renal Serum Clearances were calculated 12 hours after injection by dividing the rate of urinary excretion at this time by the total serum radiocalcium and expressing the result as per cent of the total serum in the body cleared of isotope by urinary excretion per minute For normal young animals the average value was 0 6% per minute for the normal adults it was 2 0% per minute while for the group with low phosphorus rickets the clearance was 14% or 0 16 cc /100 sq cm body surface/min

This may be compared with the values for inulin clearance or glomerular filtration rate of 0 23 cc /100 cm²/min reported by Friedman *et al*[21] and indicates that there is very little reabsorption of calcium by the tubules

The high urinary excretion and renal serum clearance in these rachitic rats despite a normal level of blood calcium suggests that there is a direct effect on calcium clearance by the kidney This may be associated with the low level of inorganic blood phosphate in these animals

Feces

The fecal excretion of radiocalcium was low in the young normal animals (see Figure 70) and almost insignificant in the rachitic group However it was relatively high in the adult animals and accounts for most of the loss from the skeleton

Conclusions

Ion exchange is undoubtedly an important factor in the uptake of radiostrontium and radiocalcium by the skeleton and is perhaps the only one of significance in the adult animal However in the young animals it does not appear to be the only mechanism The very rapid initial uptake in the normal young animals with fixation and very slow loss argues against an exchange process and in these animals much of the radiocalcium is prob-

[20] Norris W P and Kisieleski W Comparative Metabolism of Radium Strontium and Calcium *Cold Spring Harbor Symposia on Quantitative Biology* 13 164 172 (1948)

[21] Friedman S M Mackenzie K R and Friedman, C. L. Renal Function in the Adrenalectomized Rat, *Endocrinol* 43 123 125 (1948)

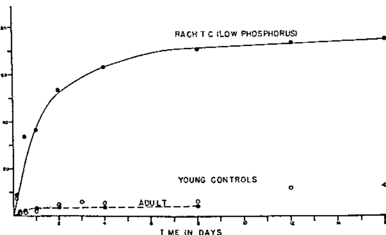


Fig 69 Cumulative Excretion of Radiocalcium in Urine (Expressed as r cent of the Administered Dose) following Intravenous Injection

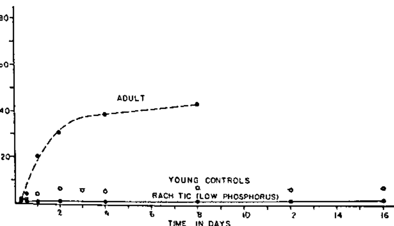


Fig 70 Cumulative Excretion of Radiocalcium in Feces (Expressed as er cent of the Administered Dose) following Intravenous Injection

ably incorporated in new bone salt In the rachitic rats the initial uptake is equally rapid but there is no fixation and the loss from the skeleton is also rapid This suggests a small and very labile calcium fraction in these animals

This labile calcium which amounts to some 15% of the total calcium in rachitic bone may represent exchangeable calcium on the surface of the bone crystals Radioautographs of femurs taken 1 hour after injection when the Ca^{45} in rachitic bone is at a maximum show the radiocalcium only in areas in which bone salt is present with none in the uncalcified osteoid matrix As the serum level of Ca^{45} continues to fall as the isotope is poured out in the urine the Ca^{45} in the labile bone fraction will fall with it since they are in equilibrium

Of particular interest is the relatively high renal clearance of calcium in the rachitic animals Since previous experiments have indicated active absorption of radiostrontium from the intestinal tract of these animals it would appear that high urinary excretion is a more important factor in the negative calcium balance in these animals than is impaired absorption from the intestinal tract

B Effect on the Metabolism of Radiostrontium

Serum

The results of these experiments with radiostrontium have been reported by Jones and Copp[2,7] Following intraperitoneal injection of $Sr^{89,90}$ the plasma level rises during the first 15 minutes as the radiostrontium is absorbed from the peritoneal cavity It then falls rather rapidly in the young and rachitic animals more slowly in the adults Indeed the plasma radiostrontium in the latter remains 5 to 10 times the value in the younger animals This may be explained by slower removal by skeleton and kidney

Skeleton

The level of $Sr^{89,90}$ in the skeleton is shown in Figure 71 As was observed by Norris and Kisieleski[220] radiostrontium rapidly concentrates in the skeleton The uptake was continuous in the adult animals up to 2 hours and reached a maximum value at 2 to 4 hours This may be accounted for by ion exchange with bone salt[22] In young animals the initial rate of uptake was five times as great and the maximum was reached within 30 minutes The radiostrontium remained fixed in the skeleton and very little loss was observed even 4 to 8 days after injection It is probable that this

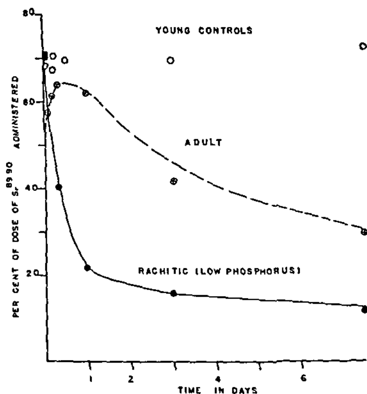


Fig 71 Per cent of the Administered Dose of Radiostrontium in the Skeleton following Intraperitoneal Injection

rapid uptake and fixation is associated with incorporation of the strontium in the newly formed bone salt

The same initial rapid uptake was observed in the rachitic animals and the maximum reached was similar However, this was followed by active removal from the skeleton, so that at the end of 24 hours, less than one third of the radiostrontium originally present at 1 hour was still left in the bones This was associated with a tremendous excretion of radiostrontium in the urine New bone salt is not formed in these rachitic animals, so that radiostrontium cannot be incorporated in this form, although ion exchange with existing bone salt may take place as it does in the adult The evidence suggests that the initial rapid uptake is due to a labile combination with bone from which the radiostrontium is readily released in the rachitic animal This may be due to exchange with labile calcium on the surface of the crystals of bone salt, or may be an initial step in the calcification process As with radiocalcium, radioautographs showed that even at the time of maximal uptake radiostrontium was deposited only in bone salt with none in uncalcified osteoid matrix

Urine

In the young animal very little radiostrontium was lost in the urine in contrast to the rachitics in which a large part of the dose was eliminated by this route within 24 hours Plasma clearances were calculated by dividing the excretion rate (determined graphically) by the radiostrontium level in the plasma Since there was great divergence in the weights of the animals in the different groups, consistency was obtained by expressing the clearance as the per cent of the total blood plasma "cleared" of radiostrontium by urinary excretion per minute, rather than the usual cc of plasma "cleared per minute

Plasma clearance was similar in both young and adult normal animals, with approximately 1 per cent of the blood plasma "cleared" per minute In the rachitic rats, the plasma clearance was 10 to 15 times greater than in the normals indicating a direct effect of this condition on the excretion of radiostrontium by the kidney This may be associated with the low level of inorganic phosphate in the blood of these animals [214]

Summary of the Effects on Metabolism of Radiocalcium and Radiostrontium

1 Radiocalcium and radiostrontium are removed from the plasma much more slowly in the adult animals than in the other two groups

2 The uptake of radiocalcium and radiostrontium by adult bone is continuous for the first 2 hours, and reaches a maximum within 4 hours

3 Skeletal uptake of radiocalcium and radiostrontium is much more rapid in the young animals, reaching a maximum within 30 to 60 minutes The deposited isotope appears to remain fixed in the bone

4 In rachitic rats the rapid initial uptake was similar to that in the normal young animals but was followed by active loss from the skeleton, so that only one third was left at 24 hours and less than one fifth at 16 days This suggests a labile combination with bone possibly due to exchange with labile calcium on the surface of the crystals of bone salt

5 A large part of the dose of radiocalcium and radiostrontium was excreted by the rachitic rats within the first 24 hours and the plasma clearance was 10 to 15 times as great as in the normal animals and approached the glomerular filtration rate This appears to be due to a direct effect of low phosphorus rickets on excretion by the kidney

Effect of a Low Phosphorus Diet on the Excretion of Radiocalcium and Radiostrontium

Day and McCollum[2,3] observed marked bone resorption and negative calcium balance in rats reared on a diet deficient in phosphorus They felt that the bone resorption was necessary to provide phosphorus for the essential needs of the soft tissues So severe was the deficiency that the animals died after 8 to 10 weeks from collapse of the softened rib cage and respiratory failure

The marked reduction in the retention of radiocalcium and radiostrontium in rachitic bone has already been discussed In the following experiments the isotopes were injected into normal young 21 day old rats or into mature adult rats and the animals were then changed to a diet low in phosphorus

A large part of the dose of Ca^{45} or $Sr^{89,90}$ is deposited in the skeleton of the normal animals within 2 to 4 hours after injection Half the animals in each group were then changed to a diet very low in phosphorus (0 01%) while the remainder were retained on the complete control diet The animals were placed in metabolism cages and the urine and feces were collected separately at daily intervals for 30 days and analyzed for Ca* or Sr* At the end of this time, the animals were sacrificed The carcass was analyzed for residual Ca^{45} or $Sr^{89,90}$

From these values the quantity of radiocalcium or radiostrontium re-

[2,3]Day H G and McCollum E V Mineral Metabolism Growth and Symptomatology of Rats on a Diet Extremely Deficient in Phosphorus *J Biol Chem* 130 269 283 (1939)

rapid uptake and fixation is associated with incorporation of the strontium in the newly formed bone salt

The same initial rapid uptake was observed in the rachitic animals and the maximum reached was similar. However, this was followed by active removal from the skeleton, so that at the end of 24 hours, less than one third of the radiostrontium originally present at 1 hour was still left in the bones. This was associated with a tremendous excretion of radiostrontium in the urine. New bone salt is not formed in these rachitic animals so that radiostrontium cannot be incorporated in this form although ion exchange with existing bone salt may take place as it does in the adult. The evidence suggests that the initial rapid uptake is due to a labile combination with bone from which the radiostrontium is readily released in the rachitic animal. This may be due to exchange with labile calcium on the surface of the crystals of bone salt or may be an initial step in the calcification process. As with radiocalcium radioautographs showed that even at the time of maximal uptake radiostrontium was deposited only in bone salt with none in uncalcified osteoid matrix.

Urine

In the young animal very little radiostrontium was lost in the urine in contrast to the rachitics in which a large part of the dose was eliminated by this route within 24 hours. Plasma clearances were calculated by dividing the excretion rate (determined graphically) by the radiostrontium level in the plasma. Since there was great divergence in the weights of the animals in the different groups consistency was obtained by expressing the clearance as the per cent of the total blood plasma "cleared" of radiostrontium by urinary excretion per minute rather than the usual cc of plasma "cleared" per minute.

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Summary of the Effects on Metabolism of Radiocalcium and Radiostrontium

1. Radiocalcium and radiostrontium are removed from the plasma much more slowly in the adult animals than in the other two groups.

2. The uptake of radiocalcium and radiostrontium by adult bone is continuous for the first 2 hours and reaches a maximum within 4 hours.

3 Skeletal uptake of radiocalcium and radiostrontium is much more rapid in the young animals reaching a maximum within 30 to 60 minutes *The deposited isotope appears to remain fixed in the bone*

4 In rachitic rats the rapid initial uptake was similar to that in the normal young animals but was followed by active loss from the skeleton so that only one third was left at 24 hours and less than one fifth at 16 days This suggests a labile combination with bone possibly due to exchange with labile calcium on the surface of the crystals of bone salt

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From these values the quantity of radiocalcium or radiostrontium re

[223]Day H G and McCollum F V Mineral Metabolism Growth and Symptomatology of Rats on a Diet Extremely Deficient in Phosphorus *J Biol Chem* **130** 269 283 (1939)

maining in the skeleton at different times was calculated and plotted on semi log paper against time The curves are shown in Figures 72 to 74

Figure 72 shows the effect of low phosphorus diet on the Ca^{45} retained by young rats By the end of the second day the excretion of radiocalcium is higher in the animals fed the low phosphorus diet and continues at a much higher rate for the duration of the experiment After two weeks the biological half life of the Ca^{45} is 37 days in the animals on the low phosphorus diet as compared to 236 days in the controls fed the complete diet The actual excretion was 5 to 6 times as great in the animals on the experimental diet

Similar curves for the adult animals are given in Figure 73 The excretion during the first week was much greater than in the young as was observed earlier However the low phosphorus diet did enhance the excretion of Ca^{45} and the biological half life was reduced from 164 to 62 days

The curves for $Sr^{89\ 90}$ in young animals are shown in Figure 74 The curves are quite similar to those for Ca^{45} and show a marked effect of the low phosphorus diet on the excretion of radiostrontium The excretion is increased almost immediately and the biological half life is reduced from 350 to 42 days

The first effect observed when an animal is restricted to a phosphorus deficient diet is a fall in the level of phosphate in serum and its disappearance from the urine The prompt increase in the excretion of radiocalcium and radiostrontium suggests that this is related to the fall in serum phosphate and may be due to the increased renal clearance mentioned earlier The increased removal from bone suggests that exchange at least of the recently deposited Ca^{45} may be affected by these changes in the phosphate level

A similar experiment was carried out in which after injection of radiocalcium the animals were changed to a diet adequate in phosphate but very low in calcium In these animals the excretion of Ca^{45} was actually reduced as compared to the controls This suggests that a low calcium diet would have little value for increasing removal of such radioactive alkaline earth metals as calcium strontium or radium from the skeleton

Summary of the Deposition of Certain Radioactive Metals in Bone

1 Many of the radioactive metals formed in nuclear fission are deposited in the skeleton The metabolism of isotopes of alkaline earth metals such as calcium strontium and barium is markedly affected by age and low

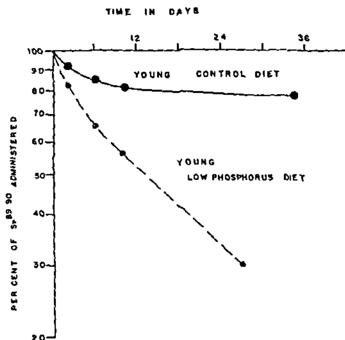


Fig 72 Per cent of the Administered Dose of Radiocalcium Retained by Young Rats Fed a Normal Diet and Those Fed a Diet Low in Phosphorus

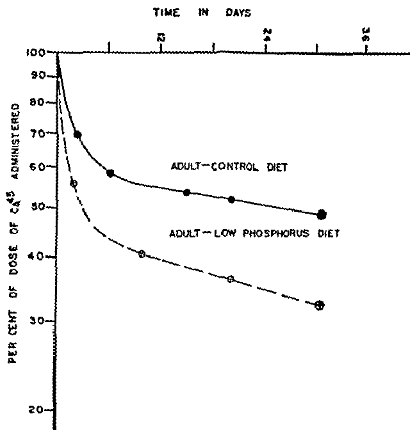


Fig 73 Per cent of the Administered Dose of Radiocalcium Retained by Adult Rats Fed a Normal Diet and by Those Fed a Diet Low in Phosphorus

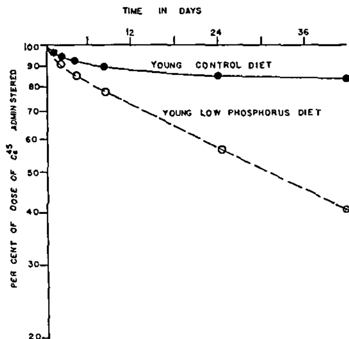


Fig 74 Per cent of the Administered Dose of Radiostrontium Retained by Young Rats Fed a Normal Diet and by Those Fed a Diet Low in Phosphorus

phosphorus rickets, while the metabolism of other heavy metals such as yttrium and cerium is largely unaffected

2 Radioautographs of the rachitic femur show that Sr* and Ca* are deposited only in the presence of bone salt, while Y* and Ce* and other heavy metals are laid down in the uncalcified osteoid matrix

3 Kinetic studies of skeletal uptake and excretion of radiocalcium and radiostrontium show considerable mineral exchange, even in adult rats Skeletal uptake is more rapid and greater in growing animals and the radioactive isotopes remain fixed in the skeleton presumably by incorporation in new bone salt

4 In rachitic rats, the initial uptake by bone is rapid, but the Ca* or Sr* *is then rapidly lost from the skeleton and excreted in urine The* "labile" fraction amounts to some 15% of the bone calcium, and may represent calcium on the surface of the crystals of bone salt

5 Renal clearance of Ca* and Sr* is 10 to 15 times as great as in the normal animals, and approaches the glomerular filtration rate This must be due to a direct effect of low phosphorus rickets on the kidney

6 When normal animals are injected with Ca* or Sr*, and are then fed a diet low in phosphorus, the excretion of the radioactive isotopes in urine is increased, and removal from the skeleton is accelerated

Conference Discussion

Follis There was vitamin D in the diet, wasn't there?

Copp There was vitamin D in the diet, just as there was in the Day and McCollum diet

Urist How much calcium was there in this diet?

Copp It was about 0 43 per cent This was a high calcium low phosphorus diet

Follis That is not too high calcium

Copp It is normal if you supply adequate phosphate

Urist This is considerably lower in calcium than in some of the diets that we have used to produce rickets

Copp If the animal has a negative phosphorus balance, then you must get a resorption of bone, so that the tissues of the body can survive You can produce in this way a very active bone resorption by the omission of only one dietary constituent

Follis This diet was composed to study uncomplicated phosphorus deficiency It was not necessarily to study rickets

Sobel But you had histological rickets?

Copp Yes depending upon how actively the animal grew At this very very low level the animals showed very little increment in weight While their bones were very thin and poorly mineralized there was little osteoid They showed the effect of starvation plus failure of bone to develop normally and calcify

If you feed a little higher level of phosphorus the animals will grow quite well and will develop quite florid rickets This diet is still quite low in phosphorus The reason we used this particular type of animal was to study a condition in which there would be active growth of bone protein but there would not be the formation of bone salt

Follis How long had those animals been on a rachitic diet?

Copp About four weeks

The serum inorganic phosphate was somewhere between 1 and 3 mg per cent The animals were x rayed before use and showed the broadened epiphyseal plate characteristic of severe rickets

Howard During that brief period had they been calcified when you had the serum calcium to 15?

Copp When we repeated the experiments with carrier free calcium or with carrier free strontium we obtained the same results Even if you raise the level of serum calcium to 15 it is still not enough to affect the rate of deposition in bone

Follis Inasmuch as there is more carbonate in rachitic bone could the calcium be combining with carbonate and not with phosphate?

Copp Some of it could be but the curve in Figure 68 represents a fraction which is actually in equilibrium with the serum

I shall discuss a theory to explain these curves in a moment

Sobel Is the serum calcium level of radioactive calcium at the end of several days the same in all these animals or higher in the rachitic animal?

Copp No actually we calculated it at several points and observed a continuous decrease Clearance measurements were made at 12 hours after injection because there is still a significant amount of Ca^{45} in the serum and you can get a fairly accurate measurement of clearance

Neuman Where did all the calcium go in the adult?

Copp Into the feces

Follis Was this calcium injected or fed?

Copp It was injected intravenously The tracer amount had no effect on the serum level of calcium The earlier Ca^{45} which had about 0 25 mg of non radioactive calcium raised the serum level to 15 to 17 mg per cent

Follis You have not given that dose of calcium that is without radio active calcium to rachitic animals and actually experimented as to whether there is an increase in ash let s say at this period when you are getting your peak change?

Copp If you give 0 25 mg Ca it is not going to affect the bone ash appreciably The radioactive tracer is a much more sensitive test of increase in calcium deposition

Follis Do you think this rise is due to the increase in serum calcium in the rachitic animal?

Copp No because it occurs just the same when you give carrier free calcium or carrier free strontium which do not change the serum calcium level

Follis It certainly does not make much sense to me—why you should get this change Does it to you?

Copp I do not know why not

It seems reasonable to me if you assume that the serum calcium is in equilibrium with the surface calcium on the crystals of bone salt When the serum calcium is tagged with Ca^{4} this radiocalcium will rapidly find its way onto the surface of the bone salt crystals By labelling the serum calcium in this way the rate at which Ca^{45} appears in bone may be measured and the relative concentration at equilibrium may be determined One may then estimate the amount of calcium in the bone which is in rather rapid equilibrium with serum calcium In a rachitic bone this labile fraction appears to be approximately 15% of the total bone calcium Figure 67 shows the initial rapid uptake of Ca^{45} which reaches equilibrium in about 30 to 60 minutes

Hastings Change in the serum calcium against time?

Copp That is shown in Figure 66 The concentration of Ca^{45} in serum falls off rather rapidly with time as does the Ca^{45} in the rachitic bone

Hastings The same way

Copp Actually, the curves are very similar from the equilibrium point on From that point, you have a parallelism between the serum Ca^{45} and labile bone Ca^{45} Both are falling rapidly because of the tremendous excretion of Ca^{45} in the urine

Shorr How about carrying it further 10 days longer?

Copp We could not measure the serum Ca^{45} at that time, but we could measure the amount in the urine

Shorr Yes

Copp We carried out an experiment on the chronic excretion of Ca^{45} which will be reported shortly

Howard Is the total calcium in the urine of these rats high?

Copp Yes

Howard That is different from human rickets

Copp The rachitic rat can absorb calcium from the intestinal tract These animals, of course, always show a markedly negative calcium balance The condition is much more severe than human rickets, because you get active bone resorption in addition to inhibition of bone mineralization

Howard In the human the Ca^{45} which you have here does not come out, and the urinary calcium is usually very low

Copp It does not come out in the normal rat

Howard In the rachitic?

Copp Yes

Follis Of course, the human is not getting vitamin D, probably, which would have something to do with the absorption of calcium whereas these animals are

Copp Also, the human is not normally a rat!

Armstrong Sometimes!

Copp Our two principal findings which we have are the labile uptake of Ca^{45} in the rachitic animal and the high renal clearance

McLean How do you feel about radioactive strontium?

Copp The differences were not quite as marked as in the case of calcium

McLean But it is the same pattern?

Copp The same pattern

Urist Dr Copp considering certain heavy metals is the deposition in bone a reaction with the organic material of bone matrix or with the inorganic salt?

Copp It is a specific property of bone matrix The reason for it is not known These metals are also deposited to some extent in soft tissues Cerium for example is found to a considerable extent in the liver

Bloom And in the spleen

Copp And in the spleen But the ultimate retention is in the bone and in the bone matrix In the case of plutonium the bone uptake is much greater

Armstrong Dr Copp I missed one point that I need a little help on The high power picture that you showed was in what circumstances?

Copp That was americium in the cortex of bone (Figure 65)

Armstrong In the cortex of what kind of animal?

Copp It was from a mature adult rat and shows the distribution in the neighborhood of the blood vessels

This characteristic spotty distribution around the blood vessels of the cortex is found with a number of heavy metals (Ce Am Cm 61) which also incidentally show very high uptake in the liver and spleen

Hastings How long after administration?

Copp I think it was a week

Follis Have you examined tissues which have a high mucopolysaccharide content for instance cornea?

Copp They do not have any The cartilage is absolutely free You do not find any in the other cartilaginous tissues

Sobel Did you produce rickets on a low phosphorus diet with the metals that did not come out of the bone subsequent to their administration?

Copp No

Sobel In other words did the bone decalcify at all?

Copp It will decalcify

Sobel But not remove the metal?

Copp But the metal is not removed

Neuman Most of these metals will hydrolyze at body pH to colloidal hydroxides and at least in part are phagocytized Wouldn t you guess that they go to the liver and spleen?

Copp They do to some very small extent Dr Bloom can answer that

Bloom There is one point I should like to make and that is yttrium plutonium, I remember distinctly, will stay at the spot of the subcutaneous injection part of it almost indefinitely and produce no pathology

Copp It does stay there The amount depends upon the form of administration

Bloom You can get a good deal At least we did

Asling It did not go well into scar tissue in some experiments that I know about

Bloom If you put it in subcutaneously and intramuscularly you will get a very nice amount for a long time

Gutman Dr Copp are the substances you injected in true molecular solution?

Copp No they must be complexed because they are quite insoluble

Gutman Wouldn t that make a difference as to how they were picked up, by the various cells of the reticuloendothelial system in those instances when macromolecular complexes were formed?

Copp Yes that is true If they are not properly complexed you will get a varying proportion going to the reticuloendothelial cells but if they are properly complexed, there will be very little

Follis Will osteoid pick these up *in vitro*?

Copp That is hard to tell because they will stick on anything

Armstrong What are they sticking to in the one I asked about with that distribution? Why is it diffuse? Why does it go where the blood vessel are? Do the little spots represent osteocytes or lacunae?

Copp The darkening on the radioautograph indicates radioactive material The strange thing is that these metals are found only around the blood vessels in the cortex I would suggest that it has something to do with the matrix, or whatever it is—the bone protein This area would be like a small endosteal surface

Armstrong Is there histologic evidence for the difference in the appearance of the bone or matrix in that area?

Copp No

Armstrong You think it is just a surface phenomenon?

Copp It will be found along the surface of the endosteum and periosteum, and also around the blood vessels

Armstrong There are two questions I should like to ask about some of the other materials Where you were able to influence calcium excretion on the part of the kidney by putting the animal on a low phosphate diet? Was the observation true that a high calcium diet will cut the rate of calcium excretion?

Copp We have not tried it I would suspect so

Armstrong One other question In your rachitic animals that have such a fast pickup and loss with your radioactive calcium, what is your idea about the other half of the calcium with your presumed adsorption, using Dr Hendricks' concept For instance, might it be the carbonate which would be influencing it by having the animal in a state of alkalosis?

Copp We never were able to get any significant effect from alkalosis or acidosis I think it would be a simple exchange with calcium of the crystal Dr Hendricks can tell you how that occurs I should like to have Dr Hendricks' reaction to this idea

Hendricks I was pressed after speaking this morning, for how much calcium is on the surface, and I think I gave the wrong answer to it I did not know immediately

Armstrong Dr Hendricks, we have some data which bear very strongly on this point and I think it may help this discussion if we could show a couple of slides which will give further information as to how calcium in the skeleton is available for turnover I will now ask Dr Singer if he will present this material and ask you to defer your discussion until later

Hendricks I shall answer the question later then

Hastings I want to hear the end of Hendricks sentence sooner or later

Armstrong We are going to ask Dr Hendricks to give several paragraphs

RETENTION AND TURNOVER OF RADIOCALCIUM BY THE SKELETON OF LARGE RATS[4]

LEON SINGER and W D ARMSTRONG

From the Department of Physiological Chemistry University of Minnesota Medical School Minneapolis Minnesota

Armstrong Dr Singer, will you present our data please?

Singer The study of calcium metabolism in the rat under various conditions through the use of radiocalcium has been conducted by several investigators[225-230] under various conditions Armstrong and Barnum[229] have presented evidence that some calcified tissues of different anatomical origin do not exchange calcium at equal rates Norris and Kisieleski[230] have reported that the femur and scapula of rats administered radiocalcium by intravenous injection reached their maximum content in approximately 100 minutes after administration and showed no significant decrease in 6 to 8 days

Methods

We have conducted an investigation to obtain data as to the turnover of calcium in selected calcified tissues over an extended period of time and to determine the per cent of the radiocalcium dose retained in the animal at various times after the administration of the radioisotope To accomplish these purposes radiocalcium (Ca^{45}) was administered to a number of large rats of the Sprague Dawley strain of comparable age and weights These animals were given intraperitoneal injections of a high specific activity

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[4]This study was supported by a grant from the Research Grants Division of the U S Public Health Service

[225]Campbell W and Greenberg D M Studies in Calcium Metabolism with Aid of Its Induced Radioactive Isotope *Proc Nat Acad Sci* **26** 176 180 (1940)

[226]Greenberg D M Studies in Mineral Metabolism with Aid of Artificial Radioactive Isotopes Tracer Experiments with Radioactive Calcium and Strontium on Mechanism of Vitamin D Action in Rachitic Rats *J Biol Chem* 157 99 104 (1945)

[227]Pecher C Biological Investigations with Radioactive Calcium and Strontium *Proc Soc Exp Biol and Med* 46 86 91 (1941)

[228]Pecher C and Pecher J Radio-calcium and Radio strontium Metabolism in Pregnant Mice *Proc Soc Exp Biol and Med* 46 91 94 (1941)

[229]Armstrong W D and Barnum C P Concurrent Use of Radioisotopes of Calcium and Phosphorus in Study of Metabolism of Calcified Tissues *J Biol Chem* 172 199 204 (1948)

[230]Norris W P and Kisieleski W Comparative Metabolism of Radium Strontium and Calcium *Cold Spring Harbor Symposia on Quantitative Biology* **13** 164 173 (1948)

radiocalcium[231] solution They were sacrificed in pairs at intervals varying from 1 to 180 days after the administration of the isotope with the exception that at 43 days and at 170 days 5 animals were killed Twenty four hours before sacrifice the animals were placed in metabolism cages and the urine collected On five of these occasions fecal collections were made at the same time In order to eliminate contamination of the excreta by spilling of the food the animals in the metabolism cages were fed a diet devoid of calcium[232-237]

The humeri the three lower lumbar vertebrae, and the epiphysis and diaphysis of the femora were taken for both chemical and radioactive analysis The four epiphyseal ends of the femurs of a single animal were pooled as one sample and the two diaphyses were united as another sample The humeri were combined as one sample as were the vertebrae The remainder of the animal not present in the selected calcified samples or the teeth constituted the carcass residue Representative aliquots of this material after homogenizing treatment were also taken for analysis The total calcium content and the fraction of administered radiocalcium remaining in each animal were calculated from the results obtained with the carcass residue and the separately examined tissues The specific activity of each sample describes the results of the division of the percentage of the injected dose of radiocalcium found in the sample by its total calcium content expressed in milligrams

Results and Discussion

In the figures which we will present, the average result obtained with each of the tissues of the animal sacrificed at each time interval is represented in graphic form In Figure 75 it can be seen that the calcium

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231 Specific activity 80 mc/gm Ca The radio calcium was obtained from the Oak Ridge National Laboratory on allocation for the U S Atomic Energy Commission.

232 Comar C L Davis G K and Singer L The Fate of Radioactive Copper Administered to the Bovine *J Biol Chem* 174 905 914 (1948)

233 Clark E P and Collip J B Tisdall Method for Determination of Blood Serum Calcium with a Suggested Modification *J Biol Chem* 63 461-464 (1925)

234 Kolthoff I M and Sandell E B *Textbook of Quantitative Inorganic Analysis* Macmillan Company New York p 347 (1943)

235 Armstrong W D and Schubert J Determination of Radioactive Carbon in Solid Samples *Anal Chem* 20 270 271 (1948)

236 Govaerts J Studies in Calcium Urinary Excretion with the Aid of Radio-calcium *Am J Physiol* 159 542 546 (1949)

237 Schour I and Massler M The Teeth in *The Rat in Laboratory Investigation* J B Lippincott Company Philadelphia, p 105 (1949)

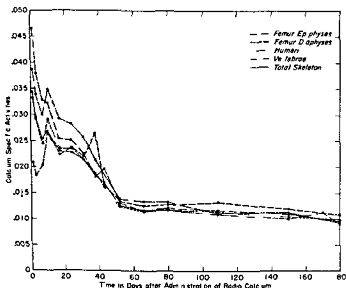


Fig 75 Changes in Calcium Specific Activity of Selected Bones and of Whole Skeleton of Rats following Administration of Radioactive Calcium

pecific activities of the selected tissues were considerably different on day 1 but decreased with time to nearly identical values in the 52nd day. The finding that within 52 days there was virtual equality of distribution of the retained radioisotope in the calcium of the separately examined bones and within the skeleton as a whole shows that an intraskeletal redistribution of the exchangeable body calcium occurs within this same period. After the 52nd day the specific activities of the calcified tissues decreased at a much reduced rate (Figure 75).

The decrease in specific activities which occurred after the 52nd day was not due to any large extent to loss of the radiocalcium by excretion. This point is emphasized by the nearly constant values for the fraction of the administered dose retained by the animals after the 52nd day to the 180th day.

Although radiocalcium was found in all samples of excreta examined the amount found after the 52nd day was insignificantly small. The average per cent found in 24 hour urine samples on 8 occasions was only 0.006%. Each of the 5 fecal collections after the 42nd day contained 0.02 to 0.03% of the administered dose. The reduction in specific activity of the skeleton and the selected tissues which occurred between the 52nd and the 180th day was due primarily to the dilution of the radiocalcium which remained nearly constant with the further increment of inert calcium gained through skeletal growth. It can be calculated that the percentage of decrease in measured specific activity of the whole skeleton (Figure 75) between the 52nd day and the 180th day was almost identical to the per cent of skeletal calcium accrued during this period. This line of reasoning leads us to the conclusion that a part of the rapid reduction in skeletal specific activities occurring before the 52nd day was also due to bone growth since 34.1% of the skeletal calcium present on the 52nd day was acquired after day 1. We have calculated from our data that 44.4% of the Ca^{45} present on day 1 was excreted by day 52 (Figure 76) and that 9.9% of the measured reduction in skeletal specific activity occurring between days 1 and 52 was due to skeletal growth and the remainder to the excretion of radiocalcium. These calculations emphasize the importance of taking into consideration in studies of the turnover rate of a radioisotope the changes in the amount of a tissue which may occur in rats in a relative short period through growth.

The data which we have presented indicate that the radiocalcium was deposited in the bones of the animals in forms which differed greatly in their relative abilities to be mobilized and to be excreted. These forms of incorporation of radiocalcium and the parts of the skeletal calcium associated with each may be referred to as the mobile fraction and the fixed

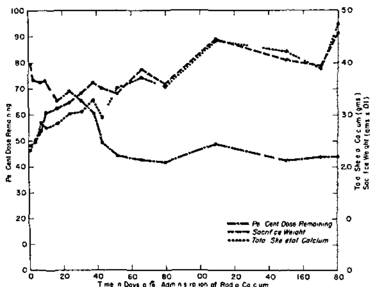


Fig 76 Body Weight and Accretion of Skeletal Calcium in Rats Retention of Radiocalcium by Rats following Intraperitoneal Administration

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The data which we have presented indicate that the radiocalcium was deposited in the bones of the animals in forms which differed greatly in their relative abilities to be mobilized and to be excreted These forms of incorporation of radiocalcium and the parts of the *skeletal calcium* associated with each may be referred to as the mobile fraction and the fixed

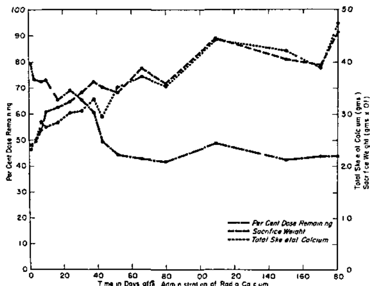


Fig 76 Body Weight and Accretion of Skeletal Calcium in Rats, Retention of Radiocalcium by Rats following Intraperitoneal Administration

fraction The fixed radiocalcium is that fraction, amounting to *circa* 45% of the injected dose which was maintained with only a slight excretion after the 52nd day It is suggested that the fixed radiocalcium was that which eventually became incarcerated by bone growth, or by rearrangement of the calcium atoms in the bone salt, in positions so remote from the body fluids as greatly to interfere with its mobilization and excretion Since the specific activity of the several bone types was maintained at quite similar values after the 52nd day (Figure 75) it can be concluded that equal weights of bone calcium had by the 52nd day, incorporated fairly uniform amounts of radiocalcium in the fixed form and that this relationship was not appreciably disturbed by further bone growth The amount of radio isotope deposited per unit weight of bone calcium differed originally among the bone types However by the 52nd day all of the easily mobile or exchangeable radiocalcium had been excreted[238] or converted into the fixed form thus reducing the specific activities of all bones to uniform values *After the 52nd day the excretion of radiocalcium occurred only from the difficultly mobilizable bone calcium* The results obtained from the urine examinations indicate that this form of bone calcium was excreted at a constant low rate

As shown in Figure 76 the calcium content of rats, exclusive of the teeth is close to 1% of the body weight in animals weighing between 249 and 405 grams

Summary

The calcium specific activities of the femoral epiphyses femoral diaphyses humeri lumbar vertebrae and the remainder of the skeleton of large rats differ soon after the administration of radiocalcium but decrease to a common value after 52 days following which the specific activities decline at equal but much reduced rates The fraction of the injected dose of radiocalcium retained in the animals declines to 42 to 45% on the 52nd day after which only small quantities of radiocalcium are excreted The results indicate the existence in large rats of two kinds of skeletal calcium which differ markedly in their rate of turnover and in the length of time over which they fix radiocalcium The apparent reduction in skeletal calcium specific activity occurring in large rats after the 52nd day following the injection of radiocalcium is due to accretion of calcium to the skeleton

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[238] The radiocalcium content of the freely exchangeable bone fractions would not be expected to be zero after the 52nd day but would correspond to the specific activity of the body fluids There is evidence (*vide infra*) that the latter specific activity was maintained at least from the 66th day onwards at a low and uniform rate

Part of the decline in skeletal specific activities which takes place in rats before radiocalcium excretion ceases is also due to skeletal growth

Conference Discussion

Singer I would like to ask Dr Hendricks what fraction he would consider to be surface calcium in this theory that he has?

Hendricks I should like you to forget what I said the first time I was asked the question so I made a guess

A more reasonable interpretation is the following though that there is an amount of calcium on the surface of the crystal irrespective of how you calculate the surface that must be approximately equal to the amount of carbonate plus the amount of citrate plus the amounts of other things there and since the citrate and other things and their equivalents are rather low to a first approximation the surface calcium would be of the order of magnitude of the surface carbonate If you will all guard me against making a slip here the carbon dioxide content will run to the order of 4 per cent of the dry weight of the bone salt—4 to 5 per cent Is that correct?

McLean A little high

Hastings On an atom it would be one of the CO_2 to 6 of the phosphate

Neuman That would be 16 per cent based on the CO_2

Hastings It would not be on the CO_2

Neuman One sixth would be 16 per cent of the total calcium

Follis On dry fat free bone it is only about 3 per cent—CO_2

Hastings No

Hendricks That is what I thought—the order of 3 per cent by weight

Hastings We are talking about two different things I am just talking about the equivalence of the calcium to the CO in atoms But never mind You go ahead

Hendricks No I want to get it right

Neuman Dry fat free bone is about 3 per cent CO but dry fat free bone is only about one half inorganic On ash it is 5 to 6 per cent CO_2

Follis That is right if you do it on ash

Hendricks I still am not certain that it will correspond to Dr Hastings figures but on that basis one would say that the order of 5 to 6 per

calcium using calcium the same as CO_3 would correspond to surface calcium, and since the total calcium content of the system is of the order of 40 per cent that would be more than 10 per cent of the total calcium of the system which would be available on the surface I mean it would be available on a surface that is covered by carbonate Of course in the teeth and possibly other portions of the skeleton the carbonate is obscured which means that that surface development is not available for exchange with external surface and that will reduce it by a considerable factor Possibly it would mean that the maximum amount of calcium that could be exchanged would be 10 per cent and the minimum amount would be on down toward the amount of the bone that is equilibrating with the external system

The suggestion now is that in Dr Copp s rachitic rats for some reason more of the surface is available for equilibration than it is in the non rachitic rats and that is the distinction between the two The way in which that could be tl e case is not that the bone salt has changed it is not that it has recrystallized but that the very process of all that is going on in the rachitic animals has made the surface more available to circulatory systems

I do not know I am not stepping out to say exactly what is the distinc tion but to indicate that in the rachitic animal even though it showed a higher turnover per cent calcium there was still not the maximum turnover with respect to calcium and that for some reason the normal animal had been prevented from approaching the degree of turnover the degree of *availability of its surface that the rachitic animal had One could imagine* ways in wh ch that is possible

Copp I think that is quite a reasonable explanation Dr Hendricks in comparing the rachitic and the adult animals where there is no increment in bone salt

There is one other factor and that is *the diffusion of the calcium into* the bone to reach the crystal surface In the rachitic animal the surface is pretty well exposed while in the adult animal the bone is quite thick There must be a definite time lag in penetration into the very solid avascular area of cortex which might account for the difference in actual uptake of Ca^{45}

The point I was most interested in was whether this exchangeable frac tion was within the limits of surface calcium

Hendricks It apparently is well within those limits by a factor of at least 2½

Armstrong It seems to me that you also have to take into account that there is a spectrum of orders and rates of exchangeability Some calcium is extremely labile but some is as Dr *Singer showed* incorporated in a situ

ation which exchanges at a very slow rate For all we know, the calcium which enters the skeleton may first be in the exchangeable fraction and later enter into the non exchangeable fraction

Singer That is quite likely

Hendricks That is this other 85 per cent In other words 10 to 12 to 16 per cent is on the surface Let's take a figure, let s take 12 Twelve per cent is on the surface, 88 is not It is the passage from the 12 per cent to the 88 that corresponds to this long preservation in the skeleton

Neuman The need for a spectrum of rates of incorporation of isotope to explain results of studies *in vivo* is a matter which deserves further mention

As we reported last year, results obtained with a powdered bone radiophosphate buffer system indicated there are indeed several processes by which isotope becomes incorporated into the bone mineral

If the material under study is organic free glycol ashed bone, isotope from the radiophosphate buffer is transferred to the mineral phase by a process which is adequately described as a "surface exchange' phenemenon The process is reversible, the final distribution of isotope between the two phases is relatively unaffected by temperature and the percent of the solid phase taking part in the exchange reaction can be calculated from specific activity considerations at the "equilibrium" point

Hastings What is that point?

Neuman This will vary with different fractions between 6 and 13 per cent, depending on pretreatment of the specimens If the samples are ashed at very high temperatures they will not show exchange, because the crystals aglomerate and the surface available for exchange is reduced

If, on the other hand, the material under study is unashed fresh powdered bone, surface exchange concepts do not adequately explain the removal of isotope from the solution by the powdered bone The system does not approach an equilibrium, rather the bone continues to remove isotope from the buffer for long periods of time Furthermore the process appeared to be irreversible These data were analyzed mathematically and were consistent with an over-all process involving two rates of incorporation of isotope a fast reaction analogous to the surface exchange observed with ashed specimens and a slow reaction which was hesitantly termed recrystallization because this second mechanism exhibited a high temperature coefficient and appeared to involve the major part of the total phosphate in the mineral phase (i e, more than just surface groups)

Since last year s meeting we have studied this problem further and have

found that the second process the slow incorporation of isotope into the center of the crystals takes place even in the dry state Obviously this is not recrystallization in an ordinary sense it would be more appropriately termed ionic diffusion diffusion through a solid crystal This diffusion process takes place at different rates with different bone specimens The most recently deposited material from metaphyseal and subperiosteal areas is the most active Where stabilization can take place with the passage of time as in old established bone the rate of incorporation is considerably less Thus we have a whole spectrum of rates of equilibration throughout the skeleton

Armstrong What is your idea on the incarceration of radiocalcium such as that which we found? I can see why radiocalcium can get into the skeleton but if the calcium enters the skeleton why can t it get out?

Neuman The rate of incorporation of isotope is always a function of the specific activity (SA) difference between the two fractions Thus if we designate three geographical positions (1) the phosphate in the extra cellular fluid (2) the phosphate in the surface layer of the crystal and (3) phosphate in the center of the crystal

the rate of movement of isotope from a to b ($_aR_b$)

$$R_{ab} = K\ (SA_a\ \ SA_b)$$

also $$R_{bc} = K\ (SA_b\ \ SA_c)$$

Shortly after the administration of isotope the rates of incorporation of isotope will be rapid because these specific activity differences are necessarily large After a few weeks however equilibrium is approached and put in terms of specific activity $SA_a = SA_b = SA$ This being the case there will be little net movement of isotope from one fraction to another The only reason isotope comes out of the bone at all is that there is a daily intake of non isotopic phosphate which lowers the specific activity of circulating phosphate However in terms of turnover of the total phosphate in the organism the amount of non isotopic material taken in and excreted daily is very small Thus the rate of removal of skeletal fixed isotope must necessarily be slow because the SA differences are small

Pfeiffer What you should do then is to put in a large amount of normal calcium which should reverse this process

Copp It actually has very very little effect

Pfeiffer I should think you could measure it

Neuman I should like to point out that in your experiment when you stopped the phosphorus intake your calcium turnover the relative dilution

of your blood specific activity was going on at a much greater rate and you lost the labile element at a greater rate too

Copp Low phosphorus is much more effective than high calcium

Neuman Yes

Copp I wanted to ask whether this recrystallization reaction could be due to incorporation of new crystals in the bone salt?

Neuman That is still another rate Crystals that are being formed at any given time should have a specific activity equal to that of the fluid bathing them In a rapidly growing animal this is not an unimportant factor In the adult animal I think surface exchange and ionic diffusion account for the gross effects that you observe As far as young animals are concerned the actual building of crystals may make up a good proportion of the total incorporation of isotope What proportions I could not say

Copp I was wondering whether since in your experiments you found so much activity in the metaphysis and periosteum the formation of new crystals might account for the irreversible reaction

Neuman The only difficulty with that idea is that there could be recrystallization in the classic sense taking place too Those particular experiments were with powdered bone and a water phase but there was no change in the phosphate concentration of the equilibrating solutions There was no net deposition of salt and therefore the uptake had to be due to an exchange or diffusion

Shorr Dr Copp you pointed out some differences between the rate of loss from bone of radiostrontium and radiocalcium This interests me practically from the point of view of an effort that I have been making to use non radioactive strontium as a means of determining turnover in bone in man rather than radioactive strontium since strontium *per se* is an element foreign to normal bone I wondered whether any inferences from the data in the human could be merely comparative one human to another or whether you think that your results invalidate considering these data a measure of the behavior of calcium

Copp We have used radiostrontium for about 8 years as the tracer for calcium While I do not like to attack the validity of such use too vigorously there is no doubt that there are quantitative differences despite the great qualitative similarity between the two

Shorr So that one could in the human use that as a standard and say, for example that this patient turns over his lime salts twice as fast as another?

Copp Yes The relative turnover of strontium is useful, but as a measure of the absolute turnover it is a little dangerous

We used large amounts of carrier free calcium and strontium in an effort to increase the excretion of radioactive strontium We found that you could give up to 100 mg of strontium to a rat and have practically no effect on excretion of the radioactive strontium

Singer I may have missed a point here but it seems to me that these experiments of yours with the very fast turnover, in the rachitic animals, of the radioactive calcium offer perhaps a unique opportunity for transferring Dr Hendricks from physical chemistry to a biological system and analyzing the shaft of such animals at the time that they are full of calcium and a number of hours later when they have lost that calcium, for getting specific information as to the type of salt that is in the surface phase—an opportunity which is pretty exciting in its possibilities, I think

Hastings We know that pretty well don t we, Dr Follis? Aren't these rachitic bones high in CO_2 relatively?

Follis Yes they have a higher CO_2 content

Singer My point is Does that follow in the character of the chain number? Does the rachitic bone have this high carbonate only during this phase?

Follis No, it is always there

Copp These turnover studies do not apply to a temporary phase, but to a steady state

Singer I am sorry That is right

Copp It would be interesting to see how much exchange there is at that 1 hour period You might remove the bone and examine the crystals to find out, by exchange reactions possibly, how much of the radiocalcium which has been taken up is on the surface

Singer And match it with C^{14} carbonate?

Copp Yes

ELECTRON MICROGRAPHY OF BONE[239]

ROBERT A ROBINSON[240]

From the Division of Orthopedic Surgery Department of Surgery University of Rochester School of Medicine and Dentistry Rochester New York

Armstrong Gentlemen it is getting late and we have several people who have information which will be of general interest in this field Dr Robinson from the University of Rochester has I think some information which was obtained from an electron microscopic investigation of bones

Robinson The fine structure of bone is of particular interest to me since I am an orthopedic surgeon The ability of bone to bend, resist torsion, compression and tensile stresses and in general act as a framework for the body depends on the fine structure or submicroscopic structure of bone Bone s resistance to stresses is offered by a combination of the mineral and organic components of which it is composed X ray diffraction studies have all suggested that the crystals of hydroxyapatite which form the hard part of bone are in the form of very tiny crystals far below the resolving power of the light microscope However it was noted that the electron micro scope has a range of resolution from a lower limit of 20 Angstroms There fore it seemed that it was theoretically possible to observe organic crystals of bone in the electron microscope The basis of the method of preparing bone and tooth samples for viewing in the electron microscope was de scribed in *Science* June 16 1950[241] The general method used was to obtain fresh bone or autoclaved bone shave the bone put the shavings in triple distilled water place the mixture in a Waring Blendor and by ten minutes blending break the bone down into fragments small enough to be observed on the electron microscope viewing screen Due to the density of the hydroxyapatite crystals it was found necessary to obtain very thin frag ments

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[239] I am indebted to Dr William Bale and Mr Michael Watson, of the Atomic Energy Project of the University of Rochester for their assistance with the electron micrography

[240] Assistant Surgeon (Orthopedics) Strong Memorial Hospital and Instructor Orthoped c Surgery University of Rochester School of Medicine and Dentistry Rochester N Y

[241] Robinson R A and Bishop F W Methods of Preparing Bone and Tooth Samples for Viewing in the Electron Microscope *Science* 3 657 (1950)

Observations on Collagen Fibers

After starting this work it was found that one could also observe collagen fibers of bone and later on in fresh specimens the amorphous cement substance was distinguished By the use of ultrasonics a more adequate dispersion of the hydroxyapatite crystals could be obtained

When fresh bone was viewed in the electron microscope fiber like structures were observed covered by an amorphous cement substance in which resided inorganic hydroxyapatite crystals Such a specimen is illustrated in the first electron micrograph (see Figure 77) At the lower corner of this first illustration one can see a collagen fiber that has been completely denuded of the surrounding cement substance and its inorganic crystals This fiber having been stripped of the other components shows the typical periodic striations of collagen fiber the periodicity of the striation being calculated at about 640 angstroms By careful observation of many such electron micrographs of fresh bone along with parallel observations on samples of autoclaved bone as is shown in Figure 78 the inorganic crystals appear to have a tabular form and in their most mature stage as found in adult cortical bone the outline of the crystals is hexagonal These crystals also appear to be long in comparison with their width and thickness The long dimension of the crystals lies usually in the long axis of the fibers as shown in Figure 77 and other illustrations of this fresh bone preparation

Dimensions of Inorganic Bone Crystals

In order to rid the inorganic crystals of their obscuring cement substance and the collagen fibers bone was autoclaved at 27 lb for two and four hours Figure 78 is an electron micrograph of such bone crystals that have been freed of their organic matrix The sample shown in Figure 78 was also resonated in a water bath using ultrasonics at approximately 400 KC In this illustration one also sees Bacillus alkaligenese These were placed in the preparation to give an idea of the comparative size of the inorganic bone crystals and a common microorganism From such electron micrographs of which Figure 78 is an example the average dimension of an inorganic bone crystal in human bone was found to be $500 \times 250 \times 100$ angstroms Preparations obtained from human cat dog and cattle source show the average size to be nearly that stated above The cattle bone tended to show slightly larger average size and the cat bone showed smaller average size than did the crystals obtained from human bone No exceptionally large crystals were ever found in bone They all fell within the general size range noted above

Identification of Hydroxyapatite

The identity of these crystals as those of hydroxyapatite was obtained

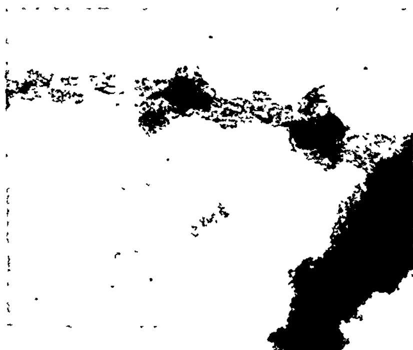


Fig. 77 Fresh Human Bone Blended

Enlargement × 34,500 1 micron = 3.45 cm 1 mm. = 290 angstrom unit

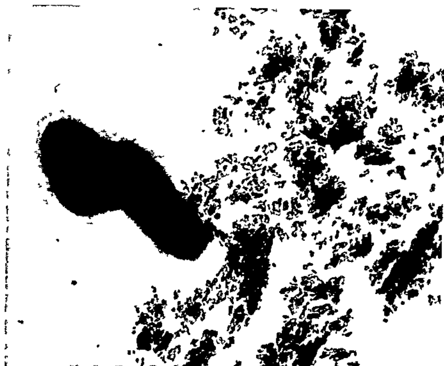


Fig 78 Autoclaved Bone, Blended and Resonated

Enlargement × 34 500 Reproduced by permission from Robinson R A and Bishop F W Methods of Preparing Bone and Tooth Samples for Viewing in the Electron Microscope *Science* 3 (No 2894) 657 (1950)

by parallel x ray diffraction studies of the samples used for observation in the electron microscope The x ray diffraction patterns obtained of those samples both of fresh and autoclaved bone were typical of hydroxyapatite Furthermore when these samples were heated to 750° and 900° C the hydroxyapatite pattern became more marked[242]

Dr Hendricks has, in a personal communication suggested that the long axis of the hexagonal tabular shaped crystal corresponds probably to the A axis of the hydroxyapatite unit cell Figures 79 and 80 are presented to show the dimension of the unit cell the general arrangements of the calcium phosphate and hydroxyl groups in the two molecules that form each unit cell and finally the arrangement of these unit cells in a stylized bone crystal This crystal shape shown in Figure 80A has been found in certain mature cortical bone In many preparations the crystals are tabular in shape but have no regular hexagonal outline However in certain samples of mature cortical cattle bone tabular crystals with a true hexagonal shape are seen These were used for the basis of Figure 80A In Figure 80B the position of the unit cell in such a crystal is outlined and the axis of that unit cell in relation to the crystal as suggested by Dr Hendricks is also noted

As discussed earlier in these transactions by Dr Hendricks the basic unit cell of enamel crystals and of bone crystals is apparently the same i e hydroxyapatite However in enamel these unit cells combine into much larger crystals than those appearing in bone Figure 81 is an electron micrograph of part of an enamel rod from a tooth As can be seen the enamel rod is made up of a fascicle of large parallel regularly arranged flattened ribbon like crystals The whole rod of which only a part is shown in this electron micrograph is apparently made up of these large hydroxyapatite crystals running with their long axes parallel to the long axis of the rod The long axis of these crystals is probably the C axis of the unit cell so that each one of the individual crystals of the enamel rod is a flat hexagon Figure 81 lends support to the crystal orientation of enamel postulated on the basis of x ray diffraction and polarized light studies[43, 46]

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[242] Brandenburger F and Schulz H R Nature of Calcification in Man and Animals and Behaviour of Inorganic Bone Substance in the Case of the Chief Human Bone Diseases *Helvet Med Acta* 12 (Supply 16) 1 63 (1945)

[243] Thewlis J X ray Analysis of Teeth *Brit J Radiol* 5 353 (1932)

[244] Bale W F Hodge H C and Warren S L Roentgen Ray Diffraction Studies of Enamel and Dentine *Am J Roentgenol* 32 369 (1934)

[245] Schmidt W J *The Elements of the Animal Body in Polarized Light* Bonn (1924) Quoted by Kitchin P C Beyond the Microscope *J Dent Res* 17 275 (1938)

[246] Orban B *Oral Histology and Embryology* 2nd Edition, C V Mosby Co St Louis (1949) [Electron Micrograph p 77 and reference relative to this picture by Boyle P E Hillier J and David n N K Preliminary Observations of the Enamel of Human and Guinea Pig Teeth Using the Electron Microscope *J Dent Res* 25 156 (1946)]

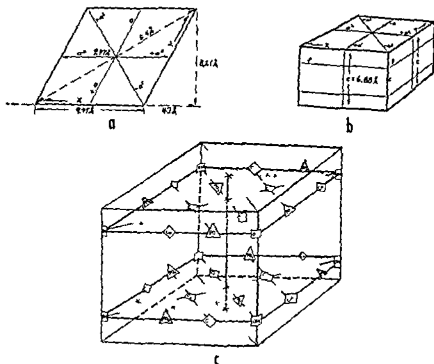


Fig 79 Diagrammatic Representation of a Bone Crystal Showing the Relationship of the Unit Cells to the Crystal (See Figure 80)

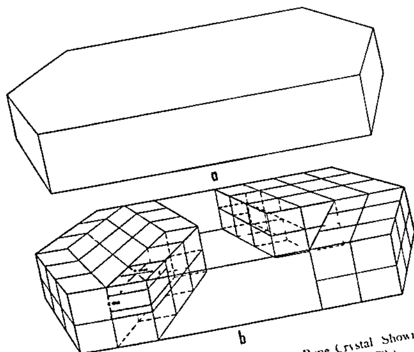


Fig 80 Diagrammatic Representation of a Bone Crystal Showing Relationship of the Unit Cells to the Crystal (See Figure 79)



Fig 81 Part of an Enamel Rod from an Autoclaved Tooth
Magnification × 34 500

Figure 82 shows an electron micrograph of enamel broken down into smaller particles. Again, the crystals are seen as thin pieces of a ribbon like appearance. These crystal fragments are much larger than any of the crystals seen in bone. They would appear to be about 600 angstroms in width, 100 to 200 angstroms in thickness and at least 5 000 or 6 000 angstroms in length. Some of these crystals have been observed to be 50 000 to 60 000 angstroms in length.

In order to determine more exactly the thickness of the hydroxyapatite crystals seen in the electron micrographs of fresh and autoclaved bone, specimens of bone and synthetic apatite having the characteristics and particle size of hydroxyapatite of bone were placed on the specimen screen of the electron microscope. After an electron micrograph of a well dispersed field of crystals had been obtained, the screen was removed from the microscope and shadow cast. The shadowing was done with uranium or chromium. The angle of the cast was 15° so that the shadow produced would be about 3 to 3.5 times as long as the thickness of the object which casts the shadow. Figure 83 shows a sample of a synthetic hydroxyapatite. Figure 84 shows the shadow cast of the sample of Figure 83. By the shadow casting technique we have confirmed the opinion gained on viewing electron micrographs of fresh and autoclaved bone that the crystals are tabular in shape, longer and broader than they are thick.

Observations of Various Preparations of Bone

Several interesting observations concerning the effect of various preparation methods of bone have been made with the electron microscope. It was found that bone boiled for fifteen minutes showed a loss of most of the cement substance while the collagen fibers were swollen in comparison with fibers seen in fresh specimens. In such a boiled sample the inorganic crystals are seen clinging on the edges of the swollen collagen fibers.

Bone from newborn animals and very old humans respond differently to the blending method of bone preparation. For example, the inorganic crystals and their binding cement substance are easily stripped from the collagen fibers from both very old and very young bone. When the fibers so stripped were stained with phosphotungstic acid, the typical periodic pattern was observed.[247]

Fresh bone treated by blending and incubation with hyaluronidase, when viewed in the electron microscope shows swelling of the cement substance

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[247]Frey-Wyssling, A., *Submicroscopic Morphology*, Elsevier Publishing Co., Inc., New York, p. 209-211 (1948).



Fig 82 Tooth Enamel Blended

Enlargement × 34 500 The blending period was longer than that used for the sample of Figure 81



Fig 83 Synthetic Hydroxyapatite Which Has Crystals Approaching the Size and Shape of Bone Crystals

Enlargement × 33 000



Fig 84 Uranium Shadow Cast of the Same Sample Used in Figure 83
Enlargement × 33 000

and dispersion of the incorporated crystals in the intrafibrillar areas whereas the crystals and the cement substance adjacent to the collagen fibers did not seem to be similarly affected by the hyaluronidase

Observations on Isotope Exchange by Bone

It has been noted in isotope exchange studies that fresh bone exchanges a larger proportion of the radioactive calcium and phosphorus than does bone from which the organic matrix has been removed Also enamel which has a very small organic matrix and large crystals showed a very small calcium and phosphorus exchange when compared with similar studies on fresh and deorganized bone Subepiphyseal or subperiosteal bone from a growing animal has the most rapid exchange of calcium and phosphorus of any of the samples tested Bone that had been heated to 700 or [illegible] C in which the hydroxyapatite x ray diffraction pattern was clarified due to probably the increased dimension of the hydroxyapatite crystal present in the preparation [319] showed a calcium and phosphorus exchange approaching that of enamel The question arose as to whether these exchange phenomena depended upon the size of the crystals of hydroxyapatite in young growing bone fresh bone bone from which the organic matter has been removed and, finally, enamel and heat treated bone By the study of subepiphyseal veal bone mature cortical cattle bone mature bone from which the organic matrix had been completely removed by glycol ashing at less than 300° C bone that had been treated at temperatures above 500 C and enamel it was possible to conclude that *the crystal size in the young growing bone was practically the same as that in mature bone that the crystal size in bone from which the organic matrix had been removed by glycol ashing were of the same size as those crystals occurring in fresh mature bone* Therefore it was concluded that *the percentage exchange of radioactive calcium and phosphorus depended solely on the size of the crystals and the surface area available for exchange on the crystal only in the case of bone from which the organic matter has been removed*

In the case of the young growing bone where the exchange was very rapid and in the case of fresh mature bone in which the exchange was moderately rapid the crystal size did not vary appreciably In fact the crystal size was the same as that in the glycol ashed bone wherein no organic matter was present Since the exchange in the glycol ashed bone was about 12 to 15 per cent this figure was taken as the exchange value representing the part of the exchange due to the surface area of the crystals

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[319] Hodge H C LeFevre M L and Bale W F Chemical and X ray Diffraction Studies of Calcium Phosphates *Ind and Eng Chem* 10 160 (Mar 15,

available for calcium and phosphorus exchange. The exchange in the fresh bone which was about 30 percent and in the subepiphyseal bone which was between 30 and 50 percent was obviously due to an additional factor connected with the organic matrix. *In the case of bone that had been heat treated above 500°, the exchange varied inversely with the increasing size of the crystal.* This was also true in the case of enamel where the organic element is at a minimum and the crystal size is large. In other words, *when organic matrix is present the calcium and phosphorus exchange is not entirely dependent upon the surface area of the hydroxyapatite crystal.*[249] In bone *the surface area of the crystal only accounts for about 12 per cent of exchange.* Anything in excess of this is *probably* due to exchangeable calcium and phosphorus ions in the matrix of the bone.

The question has been raised whether by the blending method crystals were thrown free of the collagen fibers and then returned and adhered to the fibers. Figure 85 shows a tendon collagen fiber and autoclaved bone crystals from a cat combined and blended. You can see that the crystals *do tend to stick to these fibers but not in exactly the same way as they do* in fresh bone when it is broken up in the blendor. Incidentally, autoclaved bone when prepared for four hours at 27 lb. pressure still contains 5 to 7% protein as compared with fresh bone which contains about 30% protein by weight.

Gutman: What is the difference?

Robinson: In the first place you do not see the tight banding of crystals as a close continuous incrustation right around the fiber. Rather one observes these crystals to adhere to the fibers in blocks with no particular parallelism of the fiber axis and the crystal axis. In fresh bone the crystals follow right along the course of the fibers.

Hastings: How about those other collagen fibers in the last figure? Were they treated in any special way?

Robinson: No.

Hastings: Were they clean?

Robinson: They were taken from another source and combined with crystals. They were taken from tendon and combined with crystals that were obtained from a cat.

Hastings: They look pretty barnacled to me.

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[249] Newman, W. F., and Mulryan, B. J.: The Surface Chemistry of Bone. I. Recrystallization. *J. Biol. Chem.* 185: 705 (1950).



Fig 85 Collagen Fibers from the Tendon of a Cat Combined with Autoclaved *Bone Crystals Obtained from Cat Bone* Blended

Enlargement × 34 500

Robinson Well they are but not barnacled in quite the orderly manner in which fresh bone fibers are encrusted with crystals

Hastings The fact that they stuck at all after you mixed them is the striking thing

Copp I was wondering what this variation in density means?

Robinson The crystals form a a very porous network I think that it depends on this variant Less porous areas are more opaque to the electron beam

Copp There is an overlapping

Robinson Overlapping of the crystals in some places and a thinning out in other places I suppose in this area there must be several hundred crystals

Copp We made some electron micrographs of bone by the chipping technique and obtained the very same pattern

Bevelander Did you find any definite orientation of the crystal in reference to the alignment of the fiber?

Robinson I have one picture at a high magnification of a specimen which was treated with streptococcal hyaluronidase in which the crystals in the immediate layer next to the fiber are seen to be lying on the fiber and seemed to be concentrated at nodal points of the fiber which measure 640 angstroms apart It is an unstained specimen The long crystal axes and the fiber axis in this are almost exactly parallel It appears that in the layer right next to the collagen fiber there is marked orientation But in some of the specimens I could not prove any exact orientation of the crystal and the fiber

In general in areas like this you see oriented crystals along the fiber but here is one that is lying across the fiber Of course the blending method involves disruption of the bone So I must admit that you cannot categorically state that everything you see in these pictures is just the way it existed in the fresh bone But I think the electron micrographs give a pretty good idea of what a bone collagen fiber looks like the relative size of the crystals the fact that there is cement substance in the interfibrillary space that the crystals are imbedded in the cement substance and not in the collagen fibers and finally that in general the crystals encrusting a fiber have their long axes (within 45° at least) of the long axis of the fiber

Hastings Your crystals are pretty uniform are they not?

Robinson Yes

Hastings Are they all of the same order of magnitude?

Robinson We have found them as long as 1 200 angstroms, and as short as 20 angstroms, which is the lower limit of the resolving power of the microscope In all this work we approach the lower limit of the electron microscope But most of the crystals are around 500 angstroms in length in human bone about half that in width and about one fifth that in thickness I calculated the surface area involved in one gram of hydroxyapatite with crystals of that size The theoretic specific gravity based on the molecular weight was used i e 3 099 for hydroxyapatite In this situation using crystals the size I have described (500 by 250 by 100 angstroms) we come out with a figure of 103 square meters per gram

This is very close to calculations of surface areas by gas adsorption for a gram of glycol ashed bone [39] Suppose the crystal was a cube 200 angstroms on a side with a specific gravity of 3 you come out with 96 square meters However we do not see cubes in the electron micrographs

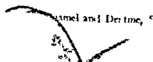
Calculating the crystals as rectangular solids 500 angstroms by 250 angstroms by 100 angstroms gave 103 square meters surface area per gram If they are 150 angstroms thick, that drops the surface area per gram down to about 81 square meters per gram

If you then calculate the number of apatite unit cells on the surface of such a crystal (Figure 80B) and divide by two (since there are two molecules in each unit cell) you come out with the fact that crystals of that size would have 12 to 13 percent of their hydroxyapatite molecules on the surface That figure happens to be very close to the amount of exchange on the surface of one gram of bone (hydroxyapatite) crystals when one is using radioactive tracer methods and when one has removed the organic substance from the bone crystals If cement substance is present (using fresh bone), then the radioactive uptake may vary from 50 percent in forming bone down to 20 percent in well formed bone

I also have a stereopticon picture of a sample and if you look through it you notice that the impression you obtained from the screen is changed slightly You can see what a porous affair this structure of little crystals can be

Conference Discussion

Follis Of course I wonder how much matrix has to do with interfering with the exchange Dr Copp showed pictures of the great ease with which



[39] Woods N A Specific Surfaces of Bone ... Enamel and Dentine, ... 103 531 (1947)

calcium can go through a very thick osteoid border In that respect bone matrix behaves just like umbilical cord and cartilage in that the diffusion back and forth is accomplished with tremendous facility

Asling Cartilage behaves in the same way There is a central core of calcification in the rib cartilage and you can scarcely get the rib out soon enough after giving an intravenous dose to avoid a concentration detectable in the radioautograph At least five minutes is the earliest that we removed the rib from the animal after injection of the tracer, and it was markedly deposited by that time

Follis That is a peculiarity of tissue carrying mucopolysaccharide

Asling No vascularity

Follis No blood vessels of course

Bezclander Are you referring to the work Schmitt did several years ago in Germany on polymerization?

Robinson If he used phalanges of the bat he could get beautiful pictures of parallel orientation of both the fiber and the crystal according to R Stuehler[251] who reviewed all that work in a lecture at Erlangen some years ago But where you have had Haversian replacement of bone and remodeling it has been difficult to demonstrate such orientation by x ray diffraction or polarized light methods except in the outer lamellar layers of the bones of large animals and the inner lamellar layers that are apparently laid down as a final touch of modeling and without Haversian replacement after the animal stops growing

Johnson There is another aspect of that which needs clarification to fully understand bone Another report would have it that in young bone the dominant x ray diffraction pattern is that of the organic component collagen With increasing age the salt component becomes more and more prominent and dominates the picture until about sixty or seventy After this age new inner rings become apparent which are not explained Warnings regarding the variations within different portions of a given bone must also be voiced with respect to variation of the same bone with age

Sobel That is the same work I am speaking of that of Meyer at Mount Sinai who is a student of Fankuchen

Dr Shorr I should just like to ask a question in connection with the apparent irreversibility of some portion of a calcium crystal

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[251] Stuehler R Ueber den Feinbau des Knochens *Fortschritte a. d. Gebiete der Roentgenstrahlen* 57 231 264 (1937)

Is it a fact that one can decalcify an adult if he lies in bed for a year or two almost completely by simple inactivation?

Shorr We studied 4 normal subjects who were immobilized in plaster casts for a period of 6 to 8 weeks They lost calcium at almost the same rate as do many fracture patients More pronounced and progressive decalcification is seen in immobilized polio patients who will continue to lose large amounts of calcium for as long as 18 months (the period studied) after the infectious episode Very pronounced skeletal rarefaction can occur in these patients Finally in osteoporosis particularly of the idiopathic type the loss of calcium from the skeleton may be so extreme that we can just distinguish the thin line of the cortex on x ray It is evident then that in these clinical states at least there seems to be almost no limit to the extent to which a previously well formed skeleton can be decalcified

GENERAL CONFERENCE DISCUSSION

Armstrong Gentlemen we have 20 minutes for free and open discussion during which time anyone can say anything that occurs to him

Urist I should like to ask Dr Robinson a question Have you reached a conclusion from studying these different preparations as to whether the crystal is associated with the amorphous or cement substance or with the fiber?

Robinson I do not find them in the fiber I found them bound *around* the fiber and tightly adherent to the periodic bands in the most proximal layer to the fiber In bone from elderly males and in two day old bone that adherence was not nearly as tight The crystals lie in the cement substance

Armstrong Just as a matter of record and for my information why do you call the particles that you see along the films crystals? It seems to me that they are particles of broken bone Have I gotten the wrong impression about this?

Robinson I consider that the blendor is a very gross instrument in relation to crystals of the size with which we are dealing Therefore I do not believe that the blade of the blendor comes around and cracks those little crystals One can use the same method of dispersion on much larger crystals of synthetic apatite and enamel and it does not break them into small units such as are observed in blended bone Therefore I think that the size observed is the size of bone crystals as they normally occur

Also the fact that the crystal size and surface area as calculated corresponds so well with the studies of radioactive calcium and phosphorus exchange and surface area as determined by gas adsorption studies makes me believe that these particles that we see are the crystals as they normally occur in bone—in other words the ultimate crystal of hydroxyapatite of bone

Shorr What is attaching them to the collagen fibers? Tricalcium citrate

Robinson One thing they are not in the collagen fiber They are in the cement substance I don t know what makes them adhere to the fiber Perhaps the cement substance next to the collagen is bound chemically to the fiber

Shorr Collagen

Hastings That is one of Hendricks surface points

Johnson I should like to get Dr Hendricks to give us some idea of the

meaning of dominance of change of pattern, please accepting that as a statement that I have taken from the literature If you have a constant surface your constant basic crystal size should be the same regardless of the age Yet in the young bone or child bone your organic pattern dominates the picture and in your adult bone the inorganic salt dominates the bone What does that mean to you in terms of mechanics of the system

Hendricks I am the wrong person to ask I do not know That was relative to this showing up of the x ray diffraction ring?

Johnson Relative to the fact that the blendor brings down a crystal that matches the surface area calculations that your studies and similar studies had led to Is it possible that this surface adsorbed material on the basic crystal can go ahead to build up a larger crystal and that would be how the more adult material could have the x ray diffraction pattern of the crystal dominant over that of the organic pattern so that you get a buildup say of thickness of the plates? You can build up your original hexagonal plates and get them thicker for instance with sandwiches of adsorbed material in between

Hendricks The old bone gave definitely larger crystals on the electron microscope

Robinson In the case of the cattle bone the average size of the crystal was about the same in developing bone (subperiosteal developing bone) and in the cattle bone that is the mature bone In the mature bone an occasional crystal was slightly larger than any seen in the subepiphyseal bone The crystal outline was smoother in the mature bone In the young bone the outline of the crystals was less exact and they looked a little bit irregular around the edges In other words I could never find the nice hexagonal crystals in the veal bone that I found in the case of the occasional larger crystal in mature cattle bone

Hendricks I can add nothing to it

Johnson Maybe that fits in with Dr Neumann's suggestion regarding incomplete lattice structure of the crystal As the lattice becomes more complete with aging your crystal would become clearer on electron microscopy

Copp Dr Robinson apropos of technique we prepared electron micrographs by actually chipping off small pieces of bone about the size of a red cell and placing them in the electron microscope. Our pictures were very similar to yours although much [illegible] this supports the validity of your method and I suspect the structure is not affected by the blending

Follis Dr Hendricks gave 100 angstroms as the size of the crystal did you not?

Hendricks Yes I was hoping that might not be remembered too exactly I think that the size would vary with the particular preparation As a matter of fact, I was using 100 Å for phosphate rock, and there it does go down as small as sixty on this other basis The francolite specimen contained crystals as large as 250 Å The hundred angstrom figure then was the order of magnitude

The indications are that these are the ultimate particles from which the material is made that you are here seeing on the electron microscope They are not in any sense artefacts of the method

Armstrong I am still confused as to what is the difference between a particle and a crystal

Hendricks A particle and a crystal?

Armstrong If we took a brick and put it in a Waring blendor and beat it up—

Hendricks You would have no more Waring blendor when you were finished

Yes, it would be a system composed of particles one of which was not like another but the particles themselves would adhere together, whereas the crystal is structurally continuous throughout Two crystals might stick together to form a particle and in this way it was attempted to reconstitute by adding external collagen fibers, calcium phosphates from another animal a cat and then stirring together Those things, agitated together as particles composed of three or four crystals together and then the whole bunch stuck down on the other, whereas in the natural material, you could see the individual crystals already dispersed out, sticking down on the fiber When you tore all the material away from the fiber, it clumped together and then went back on the fiber Apparently it was dispersed already down on the fiber and had never been taken off and allowed to clump in particles and then got back on to the fiber

Johnson I think the question here is tied up with "crystal" as defined by crystallography The point which was not apparent on the screen but which is clear on the photograph is that hexagonal crystals are visible and each axis can be seen This is quite different from what particles will show

Hendricks Dr Robinson did an injustice by projecting, because if you look at them in the original, they are extremely beautiful things and you can oftentimes pick out crystal outlines But, again you do not have to

have crystal outlines to have a single crystal It can be a rather indefinite outline and still be one continuous crystal

Robinson A crystal depends on the internal lattice structure not on its external shape

Hendricks Upon the continuity of its internal structure

Hastings You mentioned hexagonal crystals just now Is this a hexagonal crystal?

Johnson Yes

Robinson In its maturest form

Hendricks Show him some of the hexagonal plates

Robinson These things you see are enlarged about one hundred thousand times by the time we get them on the screen

Armstrong That is the point I do not understand I wish you would point out a crystal to me

Robinson You see the bigger a crystal is the more apt it is to have a well defined shape

Armstrong Yes I see now

Robinson And the older the crystal is the more apt it is to be larger

Hastings With your dimension 550

Robinson As I say these dimensions vary over a wide range After measuring many of them I came out with a figure of around 500 angstroms The width tends to be about half the length Incidentally the ratio of these dimensions remains about the same 10 5 for the length to the width and the thickness to length ratio tends to be 2 10 The thickness does not seem to vary as much as the length and the width

Johnson When you draw the crystal as seen the orientation of crystal and of collagen fiber which Dr Sobel mentioned earlier now becomes clear Dr Hendricks pointed out to me that it is the thickness of those hexagonal tablets which represent the C axis And it is the C axis which optical studies indicate is parallel to the collagen fiber Thus the major or longest axis of the hexagonal crystal will be perpendicular to the collagen fiber

Hendricks But that is not what this electron micrograph shows It shows that this long direction is parallel to the collagen

Johnson The electron micrograph does not fit with the x ray diffraction or optical polarization data?

Hendricks Yes although I think one ought not to draw any conclusion about that x ray diffraction until it is looked at pretty carefully It is the sort of a thing upon which a structure person will sometimes give a hop skip and a guess rather than a final analysis

Follis I should like to express for the record the feeling that I am sure all of us have and that is our appreciation to Dr Armstrong for arranging such an excellent meeting

Copp Seconded

Hiatt Amen (Applause)

Armstrong I was just about to point out it is now five o'clock and we have come to the end of our road I want to thank our guests for their attendance at the Conference for their excellent presentations, and for their discussion

Adjournment

JOSIAH MACY, JR.
FOUNDATION

will be glad to send a list of the conference transactions available.

These cover

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